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SECTION MEETINGS

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Biologic Decay Periods of Chloride in Man with Use of Long-Life Cl^{36} as Tracer.[*] (18371)

S. A. Threefoot, C. T. Ray, and G. E. Burch.

From the Department of Medicine, Tulane University School of Medicine and Charity Hospital of Louisiana at New Orleans.

From the data obtained in tracer studies with radiochloride,[†] Cl^{36} ($t_{.5} \cong 2 \text{ x } 10^6$ yrs.), it was possible to estimate the biologic decay periods of this element for human subjects. A knowledge of the biologic decay rates of chloride in man is of importance in the understanding of the physiologic turnover and exchange rates in normal subjects, in patients with edematous states, including congestive heart failure, and in individuals internally contaminated as a result of military and peaceful applications of nuclear energy. From previous investigations with Na^{22} in man and Cl^{36} in dogs, estimations of biologic decay rates of chloride in normal man with relatively constant compartment size were made (2-5). The observed biologic decay rates agree with the estimated values.

Cl^{36} (10,000,000 CPM or approximately

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[*] Aided by grants from the Life Insurance Medical Research Fund, a War Contract No. W-49-007-MD-389, and a Public Health Service Research Grant No. H-143.

[†] Obtained from Oak Ride National Laboratories through funds provided by the United States Atomic Energy Commission Contract No. AT-(40-1)-233.

2. Burch, George E., Threefoot, Sam A., and Cronvich, James A., *J. Lab. and Clin. Med.*, 1949, v34, 14.

3. Burch, G. E., Threefoot, S. A., and Ray, C. T., *J. Lab. and Clin. Med.*, 1950, v35, 331.

4. Burch, George, Threefoot, Sam, and Reaser, Paul, *Science*, 1948, v107, 91.

5. Threefoot, Sam, Burch, George, and Reaser, Paul, *J. Lab. and Clin. Med.*, 1949, v34, 1.

TABLE I.

Subject	Mean values in days			Injected Cl^{36} recovered, %
	$C_{.5}$*	$E_{.5}$†	$U_{.5}$‡	
Control, 501	15	16	17	96
Mild congestive heart failure, 502	19.5	38	48	69
Severe congestive heart failure, 503	45	65	169	48

* $C_{.5}$ = Time in days required for concentration of Cl^{36} in serum to reach one-half at any time after equilibrium of distribution.

† $E_{.5}$ = Time in days required for one-half of total Cl^{36} administered to be eliminated from body by all routes (Feces, urine, vomitus, and pleural and ascitic fluids were collected in these studies).

‡ $U_{.5}$ = Time in days required for one-half of total Cl^{36} administered to be eliminated in urine.

50 μc) as NaCl in water was administered intravenously to 3 female subjects: Subject No. 501, 31 years old with previous hyperthyroidism controlled with propylthiouracil; Subject No. 502, 62 years old with arteriosclerosis, essential hypertension, mild diabetes and mild congestive heart failure, who was made to compensate or allowed to decompensate, as desired, during the course of the study; Subject No. 503, 59 years old with hypertensive and arteriosclerotic heart disease with severe congestive heart failure, who died during the thirty-seventh day of study. The other 2 subjects were studied for 70 continuous days.

Blood was collected once daily under oil and immediately centrifuged; the serum was assayed as described previously(1,3,5). Urine and stools were collected as excreted and were also assayed. Any vomitus or other fluids removed, such as ascitic or pleural fluid, were likewise studied in order to determine the total elimination of Cl^{36} from the body.

The *mean* biologic decay periods for the entire study for each subject are presented in Table I. Curves for the decrease in concentration of Cl^{36} in the serum, urinary excretion (percentage of Cl^{36} not excreted in the urine) and total recovered elimination (percentage of injected Cl^{36} not recovered) for Control Subject No. 501 are shown in Fig. 1. The relationships of changes in rates to changes in intake of Cl^{35} are evident. The importance of these influences as well as the importance of changes in compartment size have been discussed previously for radiosodium(2,5) and have been applied here. The biologic decay periods for Cl^{36} varied considerably, being influenced by intake of chloride, variations in size of the chloride compartments, state and progress of the congestive heart failure, and drugs (mercurial diuretics, desoxycorticosterone acetate, bromide, sodium, and cathartics). These and other considerations will be presented in detail elsewhere.

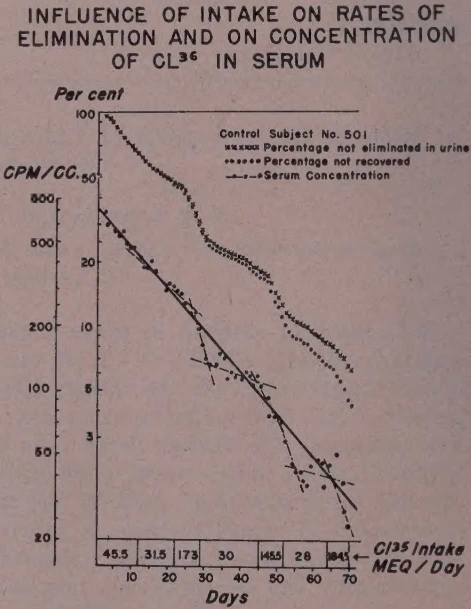

FIG. 1.

Graph of changes in concentration of Cl^{36} in the serum (CPM/cc), rate of urinary excretion of Cl^{36} (percentage of injected Cl^{36} not eliminated in the urine), and rate of elimination of total recovered Cl^{36} (percentage of injected Cl^{36} not recovered) in Control Subject No. 501. Influences of variations in intake of chloride upon these curves are indicated.

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[1] Burch, George, Reaser, Paul, Ray, Thorpe, and Threefoot, Sam, *J. Lab. and Clin. Med.*, 1950, v35, 626.

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Determination of Dextran in Blood and Urine.* (18372)

Walter Lyon Bloom and Mary Louise Willcox.
(Introduced by Arthur P. Richardson.)

From the Departments of Biochemistry and Medicine, Emory University, and the Research Division, Veterans Administration Hospital, Chamblee, Ga.

Partially hydrolyzed preparations of the bacterial polysaccharide, dextran, have been shown to be useful as non-toxic, osmotically active agents for expanding plasma volume in animals and human beings(1-3). These "derived dextrans," which are relatively unbranched α-1, 6-glucosido-compounds(4) are foreign to the body, and there is still much to be learned about their fate after injection. This paper describes a simple method for the quantitative determination of dextran in biological fluids, which is believed to be superior to the earlier methods of Hint and Thorsen (5) and Klevas(6). It is based on the fact that dextran, like other polysaccharides and unlike the simple sugars, is resistant to digestion with hot alkali and can be precipitated with ethanol.

Method. One ml of plasma, serum, whole blood, or urine is mixed with 3 ml of 30% potassium hydroxide in a 40 ml centrifuge tube, and the mixture is digested for 1 hour in a boiling water bath. Eight ml of water are added to the digested mixture, and the polysaccharide is precipitated by the addition of 20 ml of 95% ethanol, with thorough mixing. Complete precipitation of the dextran is assured by at least 3 hours' storage of the ethanol-treated mixture in a refrigerator. The precipitate is collected by centrifugation at 2500 r.p.m. for 25 minutes, and the supernatant is decanted. When whole blood is used, it is necessary to reprecipitate the polysaccharide. The precipitate is dissolved in 5 ml of water and transferred quantitatively to a volumetric flask. A final dilution is chosen such that 1 ml of solution contains from 10 to 100 μg of polysaccharide. Dextran is determined in duplicate samples of this solution, using the anthrone reagent in a method described elsewhere(7). A standard glucose solution may be used, the results being expressed in glucose equivalents.

Results. There is a linear relation between optical density and the amount of dextran in the sample over the range of 10-150 μg of dextran. The absorption spectra of the colored products of the reaction between the anthrone reagent and dextran and glucose, as determined with the Beckman spectrophotometer, are nearly identical. The absorption maximum is at 620 mμ and this wave length is chosen for colorimetry.

In samples of plasma from 13 patients who received no dextran the apparent hot-alkali-resistant polysaccharide averaged 12.4 ± 3.2 mg %. Recovery of added dextran, presented in Table I, was satisfactory.

TABLE I. Dextran Recovery from Plasma.

Dextran added, mg %	Dextran found, mg %	% recovery
480	450-456 (avg 454)	93.8-95.0 (avg 94.6)
95	94.2	99.6
19	18.6-19.5 (avg 19.3)	97.8-103.0 (avg 101.6)

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* Dextran for these studies was kindly supplied by Commercial Solvents Corp., Terre Haute, Ind.

Reviewed by the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions publlished by the authors are the result of their own study and do not necessarily reflect the opinions or policy of the Veterans Administration.

1. Gronwall, A., and Ingleman, B., *Acta Physiol. Scand.*, 1944, v1, 97.

2. Thorsen, G., *Lancet*, 1949, v1, 132.

3. Bull, J. P., Ricketts, C., Squire, J. R., Maycock, W. A., Spooner, S. J. L., Mollison, J. C., and Patterson, J. C. S., *Lancet*, 1949, v1, 134.

4. Hassid, H. Z., and Barker, H. A., *J. Biol. Chem.*, 1940, v134, 163.

5. Hint, H. C., and Thorsen, G., *Acta Chem. Scand.*, 1947, v1, 808.

6. Klevas, S., *Svensk. Kem. Tid.*, 1944, v56, 262.

7. Durham, F. W., Bloom, W. L., Lewis, G. T., and Mandel, E. E., *Public Health Rep.*, 1950, v65, 670.

TABLE II. Blood and Urine Levels of Patients Following I.V. Dextran.

Patient	Specimen	Blood mg %	Urine mg %	Total urine excretion, g
1	Control	14		
	Immed. after	715		
	24 hr	325	385	6.4
	48 ,,	121	149	3.4
	72 ,,	83	58	0.6
2	Control	10		
	Immed. after	682		
	24 hr	298	570	7.5
	48 ,,	222	495	5.9
	72 ,,	106	144	1.8

Table II records the blood and urine of 2 convalescent patients during the 72 hours following their intravenous injection with 500 ml of 6% dextran over a 30 minute period. These data are representative of a much larger series of observations made upon patients receiving dextran, and are intended to illustrate the useful application of the method to clinical studies involving the administration of dextran.

The method is not strictly specific, since it will measure the amounts of any polysaccharide resistant to digestion with hot alkali, precipitable by ethanol and capable of yielding a color with the anthrone reagent similar to or identical with that yielded by glucose. Substances meeting these requirements are not ordinarily found in significant amounts in blood and urine, so that measurements made after dextran administration may be taken with reasonable assurance that they reflect the dextran concentration in these fluids. The detection and measurement of dextran in tissue extracts containing glycogen, and possibly other polysaccharides, present another problem which is being attacked in experiments now in progress.

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Radioautographic Solubility Studies of I^{131} and P^{32} in Frozen-Dehydrated Tissues. (18373)

MARGARET W. HOLT AND SHIELDS WARREN.

From the Laboratories of Pathology, New England Deaconess Hospital and Harvard Cancer Commission, Boston, Mass.

A previous report(1) described a method for preparation of radioautographs of animal tissues without isotope loss.* This work demonstrated the ease of extraction of P^{32} from frozen-dried liver sections when they come into contact with water. This result agrees with chemical studies of P^{32} distribution in tissues which have shown that a major part of the P^{32} should be present in water soluble form(2). The present report describes results obtained when frozen-dehydrated tissue sections containing radioisotopes are exposed to extraction by a variety of reagents, both aqueous and nonaqueous. Radioautographic exposures were made with the extracted tissue sections and also with non-extracted control sections, as will be described. The results were evaluated by comparing the photographic densities and pattern of localization of radioautographs obtained from analogous tissue sections subjected to various types of extraction with those of the control sections. It is an application of the radioautographic method to obtain information about the chemical nature of radioisotopes distributed in tissues.

Solubility studies have been of very limited value in conventional technic histo-chemistry as Cain(3) has pointed out in a recent critical discussion. Tissues prepared by quick-freezing and drying warrant further investigation

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1. Holt, M. W., Cowing, R. F., and Warren, S., *Science,* 1949, v110, 328.

* With the possible exception of loss of lipoids into the paraffin used as the embedding medium. This point will be discussed later in the paper.

2. Hevesy, George, *Radioactive Indicators,* Interscience Publishers, Inc., New York, 1948, 344.

3. Cain, A. J., *Biol. Rev.,* 1950, v25, 73.

from this standpoint; however, certain limitations are inherent in all solubility studies(3). An advantage of the quick-freezing and freezing-drying process for preparing tissues consists of minimal alterations in chemical and biological characteristics of the system(4). Due to the porous solid framework that is formed, the solubility of compounds in such preparations is extremely rapid and complete, as was illustrated by Flosdorf(4) in the case of gelatin. When a thin (5 to 8 μ) frozen-dehydrated tissue section is treated with various solvents, the conditions for rapid extraction of the tissue should be optimal.

Tissues used in the present investigation were prepared and sectioned using the technics described previously(1).[†] The general procedure used in extracting the tissue sections is as follows. If the reagent being tested is not a paraffin solvent, the test is made by floating the coated section on the reagent with the cut surface down for five minutes. The section is then mounted on an albumin-coated cover glass which is placed in a vertical position in an oven at 48°C for an hour to allow firm attachment of the tissue section to the glass and to permit excess paraffin to drain off. The preparation is now ready for the radioautographic exposure or, alternatively, the paraffin may be removed from the section with xylol before making the exposure. We have not detected appreciable scattering or diffuseness of the radioautographic image due to the presence of a trace of paraffin. On the other hand, as will be pointed out later, xylol does not extract detectable amounts of I^{131} or P^{32} from the tissue sections that have been studied. If the reagent being tested is a paraffin solvent, the freshly cut tissue section is placed directly on the albumin coated cover glass (tissue side down), and the cover glass is placed in the oven as described previously. After removal from the oven, the preparation is immersed in the test solution: this removes the paraffin and subjects the tissue section to extraction simultaneously. The preparation is returned to the oven for a few minutes to aid in complete evaporation of the test solution before making the radioautographic exposure. The procedure used in making radioautographic exposures has been described previously(5).[‡]

Frozen dehydrated rat thyroid sections containing I^{131} were studied in this way. Numerous studies with conventional chemical and histological procedures have been made with this tissue and isotope. These have demonstrated that at very short time intervals (a few minutes) following the injection of I^{131}, over 90% of this isotope is present in the rat thyroid in protein bound form(6,7).

In the present investigation we used subcutaneous injection into rats of doses of carrier-free I^{131} ranging from approximately 0.5 μc/g body weight when given 4 hours before killing the animals to approximately 1.0 μc/g when given 72 hours before killing. A 3 hour radioautographic exposure of the frozen dehydrated thyroids gave satisfactory results 10 days after the injection. A large number of reagents, both organic and inorganic, were used; the solutions tested in general are those which are known to dissolve or precipitate iodine compounds (thyroxine, diiodotyrosine, and inorganic iodine) of interest in the thyroid and those which are known to cause co-

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4. Flosdorf, Earl W., See Über, Fred M., *Biophysical Research Methods,* Interscience Publishers, Inc., New York, 1950, 213.

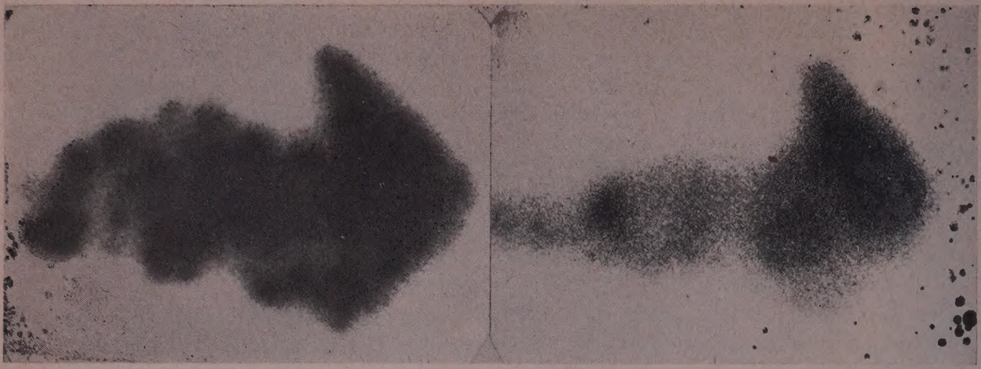
[†] We have found that, in cutting sections with this technique, the best results are obtained by painting the cut surface (by means of a camels hair brush) with paraffin at approximately 70°C. The paraffin is allowed to cool for about two seconds before cutting the section. (Sections between 2 and 8 μ in thickness are easily obtained).

5. Holt, M. W., and Warren, S., SOC. PROC. EXP. BIOL. AND MED., 1950, v73, 545.

[‡] The plastic spacers used in the present work were 10 μ in thickness; they are obtained by immersing Eastman Kodak NTB stripping film in warm water to melt off the emulsion, then stripping the 10 μ film (from which the "spacers" were cut) from the cellulose support after it has been allowed to dry. In making the exposures, we have found it useful to place a microscope slide over the cover glass before the clamp is attached in order to prevent breaking the thin cover glass.

6. Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.,* 1947, v169, 49.

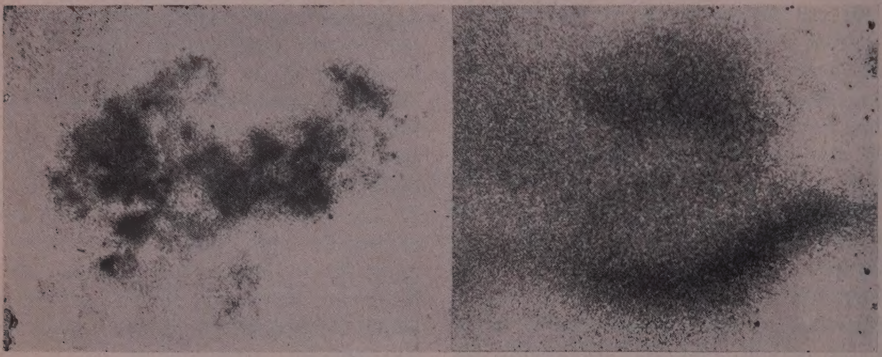
7. Leblond, C. P., and Gross, J., *Endocrinology,* 1948, v43, 306.



a. b.

FIG. 1a (left). Typical radioautograph of a section of frozen-dehydrated rat thyroid prepared without isotope loss. Areas of darkening are visible which represent localization of I^{131} in the follicles. This section was extracted with chloroform previous to the radioautographic exposure. ×50.

FIG. 1b (right). Radioautograph of an analogous section to that shown in *a* which was cut from the same tissue block. This section was extracted first with chloroform, then with distilled water before making the radioautographic exposure; comparison with *a* shows that the water dissolved out most of the isotope. ×50.



c. d.

FIG. 1c (left). Radioautograph of a thyroid section which is from a different block from those illustrated in *a* and *b*, but the thyroid tissue used for *c* was prepared prior to sectioning in the same way as the tissue used for *a* and *b*. This section was floated on saturated ammonium sulfate before it was mounted on the microscope cover glass. Xylol was used to remove the paraffin from the tissue. Comparison with *a* and *b* shows that some of the radioactive isotope was lost when the section was floated, but that the ammonium sulfate extracts considerably less of the isotope than distilled water does (the control sections on this block produced images very similar to that shown in *a*). This is typical of results observed with protein precipitants. ×50.

FIG. 1d (right). Radioautograph of a thyroid section which is analogous to those illustrated in *a* and *b*, but which was floated on distilled water before it was mounted on the microscope cover glass. Xylol was used to remove the paraffin from the tissue before making the exposure. Comparison with *a* shows that the isotope was partially washed from the tissue, resulting in a blurred image instead of the typical pattern of localization. Extraction with water usually resulted in almost complete loss of the image with the exposure times used. ×50.

agulation or denaturation of proteins. Fig. 1 (a, b, c, d) illustrates the type of results observed. Treatment with reagents which extract the radioactive material from the tissue either causes no image or a very light image to be formed on the radioautograph. In other cases, appreciable amounts of the radioactive material remained attached to the paraffin or cover glass surface, but smearing and blurring of the image showed that some extraction of the isotope from its site in the tissue had occurred. The results were first observed grossly (*i.e.* density of the image) and in addition all of the images were examined microscopically for evidences of blurring or shifting in orientation of the radioautograph pattern. The radioautograph obtained from a section which had been mounted directly on the cover glass and subjected to no solvent extraction (merely to removal of all paraffin that could be melted off the glass) served as the control in evaluating the results.

No significant differences were noted in solubility studies made on thyroids of rats killed 2, 4, 20, 48 and 72 hours after injection of the I^{131}. The results obtained may be summarized as follows: (1) The image of the extracted section was indistinguishable from the control indicating that the radioisotope was insoluble in and not displaced by the reagent (Fig. 1a). This type of result was obtained with xylol, chloroform, anhydrous ether, absolute ethyl alcohol and n-butyl alcohol. The results also indicated the radioactive isotope was partially irreversibly precipitated by these reagents. This was demonstrated by subjecting the tissue to extraction with distilled water following treatment with the precipitating agent. The radioautographs obtained when treatment with water followed the precipitation showed that the isotope was less easily extracted by water as a result of the precipitation (Fig. 1b and 1d). (2) With protein precipitants such as 5% trichloroacetic acid, Bouin's solution, 95% ethyl alcohol, acetone, 1% ferric chloride, 5M sodium chloride, saturated ammonium sulfate, and strong mineral acids and bases (1N H_2SO_4 and in 1N NaOH), careful observations of the radioautographs showed that some loss of isotope occurred, but that a major fraction of the activity was retained in the tissue section (Fig. 1c). (3) Blurring of the image and/or a lighter image was observed, indicating that the radioisotope dissolved to an appreciable extent. For example, when the tissue section was treated with saturated KI solution, although a definite radioautograph image was visible, the major fraction of the activity had been extracted. This was obvious when it was compared with the radioautographs of control sections. In this case, the activity that was not extracted was apparently that present as inorganic I^{131}. (4) Essentially complete loss of the image, indicating that the radioisotope was soluble (Fig. 1d). This type of result was obtained with reagents which, in addition to dissolving inorganic forms of iodine, would be expected to dissolve native protein (or to denature the protein but not alter its solubility). These solutions included distilled water, 1M sodium chloride, 30% urea, 1% pepsin solution, and a detergent solution (sodium lauryl sulfate).

These solubility data correlate with those to be expected if a major portion of the radioisotope were incorporated into a water soluble protein molecule which is coagulated by the common protein precipitants. If the thyroxine were present in free form in the tissue, a detectable fraction (roughly 12-27%) of the radioactivity should be insoluble in water and soluble in alkalies and butyl alcohol. The interpretation that the I^{131} present in inorganic form is a relatively minor fraction of the total and that the thyroxine and diiodotyrosine are combined in the newly formed thyroglobulin molecule correlates with the chemical analyses quoted previously (6,7). The fact that water is less effective in dissolving the isotope following treatment with protein precipitants suggests a partially nonreversible protein coagulation or fixation.

Similar studies were made on frozen-dehydrated rat tissue sections following subcutaneous injections of carrier-free P^{32} (approximately 5 $\mu c/g$). Results have been obtained at varying time intervals from injection of isotope to sacrifice of the animal. In general, the radioautographic exposure times were from 4 to 8 days using contrast lantern slides (exposures were started within one to 3 weeks following the injection).

The use of rather large doses of P^{32} was considered to be desirable in order to shorten the photographic exposure time. Studies of P^{32} distribution in tissues have indicated that with this particular isotope wide ranges of isotope dosages may be employed without altering the chemical distribution of the isotope in the various tissue components(8).

Radioautographs of frozen-dehydrated rat liver sections demonstrated that the P^{32} is distributed uniformly in the tissue. Results will be given for a twenty-four hour time interval. Densities of radioautographs of sections extracted with various solutions were compared by means of an Ansco-Sweet densitometer. In general, comparable densities were observed for control sections (paraffin not removed) and for sections treated with chloroform, ether, xylol, absolute alcohol, and acetone. Treatment with distilled water, water solutions of pepsin, urea, dilute sulfuric acid, half-saturated ammonium sulfate, and Bouin's solution decreased the density of the image to approximately one-fourth that observed for control sections (this was only slightly above background), indicating that the isotope dissolved in all of these solutions. Treatment with ether, chloroform, or acetone followed by treatment with water similarly resulted in loss of activity. Sections treated with an inorganic phosphate precipitant (slightly alkaline magnesium carbonate and calcium chloride mixture) retained a major fraction of the radioisotope, and similar results were obtained with sections treated with saturated Na_2HPO_4.

These results likewise correlate with chemical analyses(9) in showing that the radioactive material is present in water soluble and acid soluble form. The lipoid solvents do not remove detectable amounts of activity. This suggests that the lipoids were either extracted by the paraffin used for infiltration of the tissue or dispersed in the water solutions used as reagents, perhaps in colloidal form. The protein precipitants do not retain appreciable amounts of the activity in the tissue.

Other rat tissues containing P^{32} have been studied in a similar manner. These include the duodenum, small intestine, kidney, spleen, and Walker rat carcinoma. The time intervals varied between two and forty-eight hours from injection of isotope to killing of animal. With the exception of the Walker carcinoma, each of these tissues exhibits a nonhomogeneous isotope distribution. In general, the results observed are roughly comparable to those which have been described for rat liver. Images indistinguishable from those of the controls were obtained with organic solvents in general (xylol, ether, chloroform, acetone, absolute alcohol). Definite images were usually obtained with sections treated with solutions designed to retain $PO_4^{\equiv}$ (saturated Na_2HPO_4 or the phosphate reagent mentioned above) and with sections treated with protein precipitants (half-saturated ammonium sulfate, Bouin's solution). Distilled water and dilute water solutions dissolved enough of the isotope so that no image was visible when these extracted tissues were exposed for a period of time sufficient to give a satisfactory radioautograph of the control section.

The regions of selective deposition of the isotope that have been studied exhibited the same solubility characteristics as the background activity in the tissue (Fig. 2).

The possibility of the loss of lipoid material into paraffin during the infiltration of frozen-dried tissues needs further investigation. It would be desirable to use other embedding media (with solubility characteristics different from that of paraffin) for purposes of comparison.

The results which have been reported are believed to be of value in giving more information regarding the methods to be used in handling frozen-dried material with isotopes such as P^{32} and I^{131} in order to prevent isotope loss. They also apply to the handling of frozen-dried material in general, when it is desirable to avoid loss of tissue constituents. In most cases, the results agree well with data obtained from chemical analyses of similar tissues, indicating that it may be possible to utilize these methods to obtain information about the chemical nature of isotope distribution. With tissues which exhibit localized concentration of the isotope such as the glands of the duodenum and small intestine which concentrate P^{32} (Fig. 2), it is particularly

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8. Lawrence, John H., and Hamilton, Joseph G., *Advances in Biological and Medical Physics,* Academic Press, N. Y., 1948, v1, 324.

9. McCarter, J. A., and Stiljes, E. L., *Can. J. Res.,* 1948, v26E, 344.

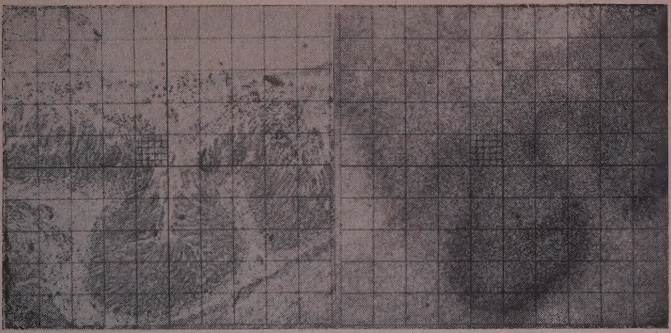

FIG. 2a (left). 5μ section of small intestine of 160 g rat prepared by quick freezing and dehydration and paraffin embedding. Animal sacrificed 4 hr after administration of 590 μc carrier-free P^{32}. Hematoxylin stain. ×50.

FIG. 2b (right). Radioautograph of a 5μ section cut from same block as tissue section shown in *a*. A 10 μ plastic ring spacer was used to separate the tissue section from the emulsion of a contrast lantern slide during the 11 day radioautographic exposure (exposure was started 10 days following sacrifice of animal). Xylol was used to extract the paraffin from the section previous to the exposure. ×50.

interesting to compare the properties of the localized isotope with those of the background activity in the tissue. It is possible that if the localized P^{32} is different chemically, further tests designed for specific differential precipitation might give information about the nature of the compounds present in a given location. Our results have shown that inorganic phosphorus and proteins can be precipitated *in situ* in the tissue by use of the proper reagents. After the tissue section is mounted, treatment with the proper fixative should not cause loss of the test material (the results have shown that acetone, for example, could be applied without loss of I^{131} or P^{32}); however, the effectiveness of the fixative is questionable since the results have shown that precipitation by such reagents is only partially irreversible. In testing for a given material in frozen-dried tissue sections, the procedure to be followed should be (1) Cut and mount the tissue section without contact with any reagents; (2) Remove the embedding medium with a solvent in which the test material is insoluble; (3) Apply a reagent designed toward specific differential precipitation of the test material, and (4) Employ other procedures (specific stains or radioautographic exposures) which make it possible to visualize the test material.

One of us (M.W.H.) wishes to express appreciation to the Atomic Energy Commission for a National Research Council A.E.C. Postdoctoral Fellowship in the Biological Sciences, which made this research possible. A portion of this work was done under Atomic Energy Commission contract number AT (30-1)-901 with the New England Deaconess Hospital. The isotopes used were supplied by the Oak Ridge National Laboratory, Oak Ridge, Tenn. We also wish to express our appreciation to Miss Alice A. Williams for technical assistance in part of this work.

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Thermal Coagulation Point and Iodoacetate Index as Detectors of Abnormal Serum Proteins and Underlying Illness. (18374)

George B. Jerzy Glass, Linn J. Boyd, and I. J. Dworecki.
(Introduced by A. L. Copley.)
From the Department of Medicine, New York Medical College, Flower and Fifth Ave. Hospitals, New York City

Frequent abnormality of the thermal coagulation of the blood serum was first described in patients with malignancies(1). Since a higher temperature was required to coagulate cancer sera to a clot not susceptible to liquefaction from standardized shaking than normal sera, the gel of the sera of cancer patients appeared looser than normal. The phenomenon was not specific for cancer, since a similar abnormality was encountered in pregnancy, nephrosis, pneumonia, severe tuberculosis, Hodgkin's disease, heart failure and malnutrition. As additional cases were studied(2), it became evident that this abnormality did not constitute a cancer test but was a nonspecific serological "signature of the gravity of the disease," irrespective of the neoplastic or non-neoplastic etiology. Thus, the one-year mortality among 628 patients was 10 times as great in those with highly abnormal thermal coagulation as in those with normal coagulation(2). Apart from noting that this phenomenon depended upon quantitative and qualitative disturbances of serum proteins, particularly serum albumin(1,2), the results of 1500 determinations of the serum thermal coagulation point in a variety of diseases indicated this test had value in the detection of organic disease, an application to prognosis, and possibilities for studying disturbances of serum proteins(3). The existence of the abnormal thermal coagulation of serum in patients with cancer and some severe non-neoplastic diseases was also demonstrated by measurement of the least coagulable serum concentration(4). Krzeminska-Lawkowicz determined the least dilution of serum at which an unbreakable serum clot formed on heating. On the basis of 145 determinations in normal and pathological sera(4), she confirmed the observations mentioned above and concluded that sera of patients with cancer and some severe non-neoplastic diseases required less dilution to prevent thermal coagulation of serum than those of normal subjects or patients with other disease. The frequent occurrence of the abnormal thermal coagulation with normal total protein content, and its dependence upon qualitative changes in serum proteins, was also stressed(1-4). Other authors(5,6) tried to employ the principle of abnormal thermal coagulation of serum for screening cancer. They also determined the least coagulable serum concentration with a modified method and, in addition, measured the iodoacetate index, *i.e.* the smallest concentration of iodoacetate per unit of serum proteins sufficient to prevent the heat gelification of serum. The conclusions drawn by Huggins *et al.* could not be confirmed by other workers(7-13) who found iodoacetate test unsatisfactory for the

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TABLE I. Correlation of Thermal Coagulation Point of Blood Serum and Iodoacetate Test in 219 Pathological Sera.

Micromolarity of iodoacetate necessary to prevent thermal coagulation	No. cases examined in each group	% distribution in each group of thermal coagulation points				
		75-81	82-84	85-89	90-95	95°C
39-36	128	84.4	13.3	2.3	0	0
33-30	49	49.0	44.8	4.1	2.1	0
27-24	24	16.6	36.6	21.8	12.5	12.5
18	18	0	11.1	11.1	33.4	44.4

diagnosis of cancer and as a screening test for malignancy. More recently, Huggins *et al.* have altered their viewpoint as indicated by their recent statement of the significance of defective thermal coagulation as a "screening device for protein metabolism" and an "aid in detection of serious disease"(14) instead of a "screen for cancer" or "useful information in recognition of cancer in man" (5,6).

The clinical importance of the phenomenon of abnormal thermal coagulation of serum raises the problem of whether the thermal coagulation should be measured by the determination of the thermal coagulation point, a method which supplied valuable data concerning its clinical significance and mechanism, or by the technics recently introduced. Since no data based on simultaneous determination are available, it was of interest to compare the data obtained by the use of both technics in the same pathological sera. Moreover, since the technic of measurement of the thermal coagulation point was quoted recently(14) as an abandoned method yielding results of limited value, an evaluation of the merits of recent technical improvements for the study of thermal coagulation seemed appropriate.

Material and methods. Two hundred and forty sera were collected under fasting conditions, with the precautions described previously(3).* They were obtained from 51 cancer patients and 189 individuals with various organic, non-neoplastic, diseases. The non-neoplastic cases had, for the most part, diseases which previously were found to be associated often with abnormal thermal coagulation of serum(1-3). The diagnoses were verified by all means possible, and in cases of cancer they were confirmed by operation, biopsy or autopsy. The iodoacetate test was performed by the Huggins-Miller-Jensen technic(15), the total protein content by the method of Greenberg(16), and the thermal coagulation point of serum by the recent simplified technic of Jerzy Glass(3).

Correlation of both technics. Data were obtained with both technics in 219 pathological sera (Tables I and II, Fig. 1). The serum protein content need not be considered in comparing these results since it is the same with both technics, as will be discussed below. The data reveal an inverse relationship between the level of the thermal coagulation point and the concentration of iodoacetate sufficient to prevent the heat coagulation process. The higher the temperature necessary for heat coagulation, the less iodoacetate is required to prevent the coagulation of serum, if temperature of heating is kept constant, and *vice versa.* This is demonstrated

TABLE II. Average Concentration of Iodoacetate Necessary to Prevent Thermal Coagulation of Blood Serum at Various Levels of Thermal Coagulation Point.

Thermal coagulation point, °C	No. cases examined in each group	Avg conc. of iodoacetate in micromols, calculated with stand. error
75-81	136	36.6 ± 0.1
82-84	50	32.0 ± 0.6
85-89	12	29.7 ± 4.1
90-95	10	21.6 ± 2.3
>95	11	19.9 ± 1.0

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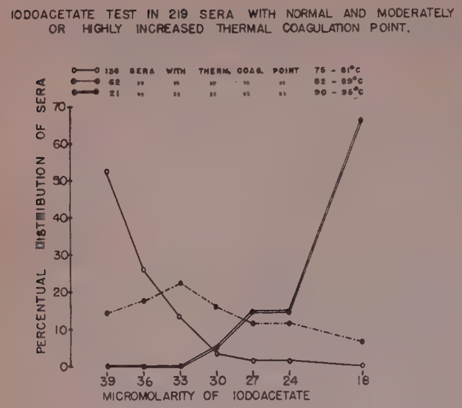

FIG. 1.

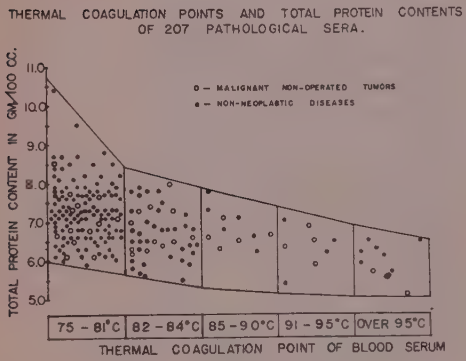

FIG. 2.

most clearly in sera showing normal thermal coagulation, as well as in those presenting a highly defective coagulation (Fig. 1, Table I). It is true also for sera showing a slightly defective thermal coagulation (thermal coagulation point of 82-84°C and iodoacetate concentration of 30-33 micromoles). There is a frequent dissociation, however, between results of individual tests performed with both technics on sera with a moderately abnormal coagulation, *i.e.* thermal coagulation point of 82-89°C and iodoacetate concentration of 24-27 micromoles. With iodoacetate values of 24-27 micromoles, the thermal coagulation point may be normal or slightly, moderately or greatly increased (Table I). That the reverse is true at moderately increased thermal coagulation points is shown in Fig. 1. The average values of both tests still show an inverse relationship, however, even at that level of thermal coagulation as listed in Table II.

Calculation of thermal coagulation defect in terms of total serum proteins. The relationship just described should also hold if both tests were calculated in terms of the relation of thermal coagulation point to total serum proteins and iodoacetate index. From the diagnostic point of view, this feature would be important, if there were statistical evidence, as contended previously by Huggins *et al.*(5), that cancer sera required less iodoacetate on the average per unit of weight of serum proteins than non-neoplastic sera. Because of the inverse correlation between the iodoacetate concentration and the thermal coagulation point of serum it could be presumed that the same phenomenon would also be evident by a higher position of cancer sera in a correlation scatter diagram, if the thermal coagulation points of neoplastic and non-neoplastic sera were plotted against their total protein content. These data (Fig. 2) indicate, however, that the cancer sera do not differ from non-neoplastic sera in this respect, confirming earlier studies(3). From the diagnostic point of view, therefore, the calculation of defective thermal coagulation in terms of total protein content of serum, is not of additional value in cancer diagnosis.

Thermal coagulation point and iodoacetate index of serum in disease. Data obtained simultaneously with both tests in 240 sera of patients with cancer and non-neoplastic disease are summarized in Table III. The abnormal iodoacetate index (below 9.0) (5,17) occurred in 32.4% of non-operated malignant tumors, and in 23.2% of non-neoplastic diseases. The superposition of the postoperative state upon the cancer, both of which increase the thermal coagulation defect (3,9), resulted in an increased frequency of abnormal iodoacetate indices in the group of patients studied shortly after operative removal of the malignant tumor, and contrasted with a normal iodoacetate index in patients studied long after a successful operation. The abnormally low iodoacetate indices in diseases

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TABLE III. Iodoacetate Index and Thermal Coagulation Point of Blood Serum in 240 Patients with Neoplastic and Non-neoplastic Disease.

Diagnosis	Iodoacetate index			Thermal coagulation point		
	No. cases studied	No. abnormal tests*	%	No. cases studied	No. abnormal tests*	%
Neoplastic diseases:						
A. Non-operated tumors						
Digestive tract, kidney, bladder, prostate, lung, thyroid	25	9	36.0	23	9	39.1
Cervix, uterus, breast, skin, bones, and brain	12	3	25.0	12	3	25.0
Total	37	12	32.4	35	12	34.3
B. Operated tumors						
<2 wk after operation	9	5	55.6	8	4	50.0
Several mos. or yrs. after successful operation	4	0	0.0	5	1	20.0
Total	13	5	38.5	13	5	38.5
Non-neoplastic diseases:						
A. Prone to defective thermal coagulation of serum†	146	39	26.7	138	31	22.5
B. Other misc. diseases‡	44	5	11.4	34	3	8.8
Total	190	44	23.2	172	34	19.8
Total of cases	240	61	25.4	220	51	23.2

* As definitely abnormal tests were considered iodoacetate index below 9.0 and thermal coagulation point increased 2-4+, *i.e.*, above 83°C.

† Non-neoplastic diseases listed in this table and reported in detail elsewhere(11) included pneumonia, pyogenic processes, pulmonary tuberculosis, complicated by infection or hemorrhage pregnancy, heart failure, postoperative states, ulcerative colitis, pyloric stenosis, and obstructive jaundice.

‡ Other miscellaneous diseases included pleurisy, infectious hepatitis, peptic ulcer, anemias of various origin, diabetes mellitus, bronchial asthma, peptic ulcer, early pregnancy, etc.

not prone to abnormal thermal coagulation (3) occurred in only 11.4% as compared to 26.7% of abnormal indices in the group of patients prone to this disturbance (Table III). The abnormally increased thermal coagulation point of serum (2-4 plus, above 83°C) was found in 34.3% of cases of non-operated cancer, 22.5% of cases of non-neoplastic disease prone to thermal coagulation defect, and in only 8.8% of other miscellaneous diseases. With this technic, also, the occurrence of abnormal thermal coagulation in recently operated cases of cancer was very frequent, and with both technics the abnormality in thermal coagulation was more frequently detected in cancer of the digestive and urogenital tract, than in tumors of genital organs, breast, skin or brain. It is evident from Fig. 3, which compares the distribution of the iodoacetate index and thermal coagulation point of blood serum in neoplastic and non-neoplastic diseases, that neither of these technics discloses a significant difference between the two groups of cases. Although the increased thermal coagulation point and low iodoacetate index (5.0-6.0) were noted with slightly greater frequency in cancer than in non-neoplastic disease, statistical evaluation of these data shows that this difference is not significant. The simultaneous determination of the thermal coagulation defect with both technics is of little additional value. Of 207 sera, including 35 patients with non-operated cancer and 172 with non-neoplastic disease, defective thermal coagulation (as indicated either by an iodoacetate index below 9.0 or a thermal coagulation point above 83°C or both) was found in 61 instances (29.4%). Since 15 sera were from cancer patients and 46 from those with non-neoplastic disease, the percentage of abnormal thermal coagulation in cancer as detected by one or the other, or both, tests was 42.9% as compared to 26.8% in the non-neoplastic group. Using the "4-fold table" and calculating the χ^2 value by the corrected Yates formula, one obtains $\chi^2 = 2.9$, at $n = 1$, and a probability value, P, of between 0.1 and 0.05. This indicates no statistically significant difference in thermal coagulation between both groups of dis-

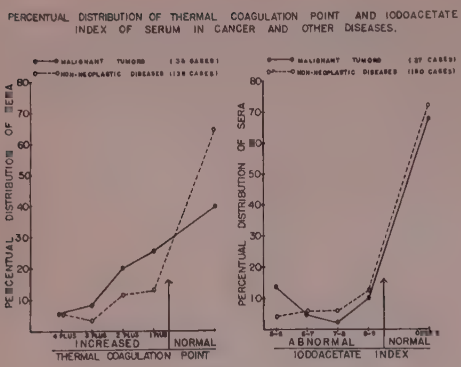

FIG. 3.

eases.

The simultaneous use of both technics slightly increases the percentage of detected abnormalities in thermal coagulation in all pathological cases as shown by the fact that 25.4% were detected with the iodoacetate index, 23.2% with the thermal coagulation point, and 30.4% with both technics simultaneously.

Comment and conclusions. The simultaneous determination of thermal coagulation of blood serum in a large series of cases by the measurement of thermal coagulation point and iodoacetate index has shown similarity of results. There was an inverse relationship, in most instances, between the level of the thermal coagulation point of serum and the concentration of iodoacetate necessary to prevent heat coagulation. The dissociation between the results of both technics appeared exclusively in the group of cases showing a moderate defect in thermal coagulation. This could have resulted, in part, from calculation of the thermal coagulation defect, in the case of the iodoacetate index, in terms of total proteins content, and the omission of the protein factor in the estimation of the thermal coagulation point of serum. It must have been due at times, however, to the results obtained with the iodoacetate technic, which, for unknown reasons, are not always reproducible(7,9). The percentage of abnormalities found in the pathological sera with both technics, singly or combined, in cancer cases and non-neoplastic diseases, was very similar. Neither technic permits a sharp differentiation between neoplastic and non-neoplastic disease. Since abnormal thermal coagulation seems to be related to decrease in sulfhydryl groups of serum proteins which occurs in cancer(18), as well as in metabolic and infectious diseases(19), there is no theoretical basis for the use of measurement of defective thermal coagulation as a diagnostic test for cancer.

The calculation of the thermal coagulation defect per unit of serum proteins, as in the Huggins technic, has not added to the specificity of this method for differentiation of neoplastic from non-neoplastic diseases, nor changed the total percentage of serological abnormalities in pathological material tested. It is possible, however, that the employment of two or more technics for determination of abnormal structure of serum gel may increase the percentage of pathological sera detected. This occurred when the serum gel was tested simultaneously by the measurement of the thermal coagulation point, and the determination of the speed of diffusion of electrolyte through the gelified column of serum(20).

Quantitative disturbances in serum proteins can be detected by many non-specific methods, including serological flocculation or turbidity reactions and sedimentation rate. Determination of qualitative alteration within individual fractions of serum proteins requires much more complicated technics(21-24, 2,6) or study of the electrophoretic pattern of serum. The chief advantage of the method of examination of thermal coagulation of serum is that it provides a very simple way

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to detect certain quantitative and qualitative alterations, which frequently escape other non-specific serological tests, which are dependent upon different abnormalities in proteins. Thus the presence of disease is easily discovered and diagnostic and prognostic conclusions may be drawn(3). Measurement of the thermal coagulation of serum with determination of the thermal coagulation point is simpler than other technics. It has a smaller intrinsic error because it does not require an additional measurement of protein concentration, nor the calculating of a ratio. The results obtained by either method are equally significant for the detection of abnormal thermal coagulation; the scope of both is determined by factors inherent in the basic principle.

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Effect of Antibiotics on Intestinal Microflora and on Growth of Turkeys and Pigs.* (18375)

JOHN McN. SIEBURTH, JOSE GUTIERREZ, JAMES McGINNIS, JOEL R. STERN, AND B. H. SCHNEIDER.†

From the Department of Poultry Husbandry, State College of Washington, Pullman

A marked increase in growth of turkey poults fed a diet supplemented with a whole dried aureomycin fermentation mash was reported by McGinnis *et al.*(1). This response was obtained under conditions where vitamin B_{12} was ineffective in promoting growth. Stokstad, *et al.*(2) reported that a similar product gave increased chick growth over that obtained with crystalline vitamin B_{12}. Subsequent reports have shown that crystalline aureomycin(3,4) and streptomycin(5) were effective in promoting growth of chicks, turkeys and pigs. Unpublished data from this laboratory confirm these reports and have also shown that penicillin, terramycin, bacitracin and 3-nitro-4-hydroxybenzene arsonic acid were effective in promoting growth of turkey poults.

Several possible explanations have been offered for the growth-promoting properties of antibiotics: (a) the inhibition of toxin-producing bacteria; (b) the reduction in total numbers of intestinal bacteria and lowering of competition between host and microflora for nutrients; and (c) the selective inhibition of a group of the microflora permits increased growth of other microorganisms that synthesize unidentified essential nutrients. The experiments described in this paper were conducted to determine the effects of antibiotics on the intestinal microflora of turkey poults and pigs and to find whether the changes could be correlated with increased animal growth.

Procedure. The turkey poults used in these studies were kept in electrically heated wire-floored battery brooders and were fed the experimental diets *ad libitum* immediately after hatching. Drinking water was available in metal pans at all times. The basal diet was composed of ingredients ordinarily used in making practical feeds; its composition is given in Table I. Turkey poults fed

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TABLE I. Composition of Basal Diet.

Ingredients	Parts
Ground corn	45.
Soybean oil meal (solvent)	39.
Fish meal	10.
Dehydrated alfalfa meal	2.5
Limestone	2.
Dicalcium phosphate	1.
Salt	.5
Riboflavin concentrate (500 μg/g)	.5
Vit. A & D supplement (5000 A, 1000 D/g)	.2
Choline	.25
$MnSO_4$*	—

* 8 g/100 lb.

diets supplemented with 15 p.p.m. of penicillin G (K salt) or terramycin and a combination of these antibiotics were sacrificed and the cecal contents removed for enumerating some of the various bacterial types. Even though this level of antibiotics gave a marked increase in poult growth, no consistent and reproducible differences in the microflora being enumerated could be obtained. When the level of antibiotic supplementation was increased to 100 p.p.m., it appeared that some differences were obtained.

In Exp. 1, turkeys were sacrificed after the second and third weeks and the cecal contents removed for counting of bacteria present. The cecal contents of 5 poults were thoroughly mixed and a 1 ml aliquot was used for serial decimal dilutions which were plated in triplicate on the following media:

1. Eugonagar (BBL)—for "total anaerobes" and "total aerobes". 2. Violet red, bile agar (Difco)—for Coliforms. 3. SS agar (Difco)—for lactose negative, gram negative rods.

A set of plates on medium 1 was incubated under aerobic and a duplicate set under anaerobic conditions using the procedure of Weiss and Spaulding(6) (with the inclusion of 10% CO_2) to give an index to the total number of "aerobes" and "anaerobes". The plates for coliforms and lactose-negative rods were incubated for 18-24 hours under aerobic conditions at 37°C. Five tubes of the "SF" medium of Hajna and Perry(7) were inoculated with each dilution and incubated 36 hours at 45°C for the enumeration of enterococci. The most probable numbers were determined from the tables of Halvorson and Ziegler(8). The poult growth data and bacterial counts on cecal contents at 2 and 3 weeks of age are given in Table II.

The number of bacterial types found in the cecal contents was influenced by the antibiotic supplements, but the effects of penicillin and terramycin were not consistent even though both antibiotics gave increased poult growth. In general, the coliform count was lowered. One of the most interesting observations was the increase in lactose-negative bacteria in the ceca of birds fed terramycin. In a subsequent trial, a greatly increased number of such bacteria was also found in ceca of birds fed penicillin. A more thorough examination of the lactose-negative colonies from SS agar demonstrated that members of the genus *Proteus* were present. These colonies were seeded on Kliglers iron agar and found to be H_2S producers which exhibited a strong urease activity as indicated by the medium of Rustigian and Stuart(9).

The presence of *Proteus* in ceca of birds fed the basal diet could not be demonstrated by using the urea ricinoleate medium of Zarett and Doetsch(11). In contrast, when this medium was used to plate cecal contents of poults fed antibiotics *Proteus* could be recognized and enumerated after 24 hours incubation at 37°C. This confirms the results of Harrison and Hanson(12) who did not find *Proteus* in the cecal feces of turkeys which were probably fed a diet without antibiotic supplementation. The change in flora, indicated by the presence of *Proteus*, that followed the administration of terramycin appears to be similar to that caused by aureomycin, since Metzger and Shapse(10) found an immediate and marked increase of *Proteus* in

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TABLE II. Exp. 1. Effect of Antibiotics on Turkey Growth and Microflora of Cecal Contents.

Supplement to basal diet			No. of bacterial types in cecal contents (millions per ml of sample)				
Penicillin, p.p.m.	Terramycin, p.p.m.	Avg poult wt, g	Total anaerobes	Total aerobes	Coliforms	Lactose negative rods	Enterococci
			2 wk				
—	—	142(31)*	7170	663	126	.0002	171
100	—	191(34)	8200	69	9.3	.0002	5.42
—	100	191(48)	4730	66	4.9	.016	349
100	100	198(46)	4630	—	6	.68	349
			3 wk				
—	—	287(26)	323	69	16.6	.000	1.09
100	—	389(29)	6600	167	.4	.0004	.0493
—	100	359(38)	1213	450	4.9	.079	17.1
100	100	410(35)	3130	910	63.0	7.2	91.8

* No. of poults in parentheses.

TABLE III. Exp. 2. Effect of Penicillin and Terramycin on Growth and *C. perfringens* Counts of Cecal Contents.

Supplement to basal diet		Avg wt, g		Counts of black colonies/ml	
Penicillin, p.p.m.	Terramycin, p.p.m.	7 days	14 days	7 days	14 days
—	—	84(21)*	170(9)	3800	25230
100	—	95(22)	246(11)	<100	<100
—	100	96(28)	229(11)	<100	<100
100	100	103(26)	244(11)	<100	<100

* No. of poults in parentheses.

human intestinal contents following administration of aureomycin.

Proteus did not always increase following the feeding of antibiotics to turkeys. It therefore does not appear that the growth-promoting effect of antibiotics was due to an influence on *Proteus* alone, if at all.

Since clear-cut results were not obtained in Experiment 1, a second experiment was conducted. Because the possibility existed that antibiotics promote growth by preventing the production of bacterial toxins in the intestine, it was decided to make counts of certain *Clostridium* in the cecal contents of turkeys fed the basal diet with and without antibiotics. For this purpose a sulfite-glucose-iron agar medium was used which had previously been employed by Wilson and Blair(13) and Skinner and Baskin(14) for counting *Clostridium perfringens (C. welchii)* in water and in soils, respectively. The plates of this medium were incubated anaerobically at 37°C for 12-18 hours. After this time, large black colonies were counted. Typical colonies were inoculated into milk and were found to produce "stormy fermentation" characteristic of *C. perfringens*. This evidence along with the experience of others indicates that these organisms probably belong to the genus *Clostridium* and may possibly be *C. perfringens*. The results on poult growth and *C. perfringens* counts are given in Table III.

Following the observations that penicillin and terramycin inhibited the growth of *C. perfringens* in the ceca of turkeys, samples of feces were collected from pigs fed a purified diet with and without terramycin supplementation. A count was made of *C. perfringens* and the results are presented in Table IV. The average weights of pigs fed the diet are also given.

The reduction in the number of *C. perfringens* brought about by terramycin was

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TABLE IV. Effect of Terramycin on Growth and *C. perfringens* Count of Pig Feces.

Supplement to basal diet			Counts of black colonies/g	
Vit. B$_{12}$, g/kg	Terramycin. mg/kg	Avg wt at 8 wk, lbs.	1st count	2nd count
—	—	57.0	—	33700000
50	—	66.3	34300000	79400000
50	15	75.8	10	10

* 6 pigs/group.

very marked. A second count showed even more striking results. These findings that penicillin and terramycin inhibit *C. perfringens* agree with the results reported by Bliss and Warth(15) who showed penicillin and aureomycin to be inhibitory to members of this genus *in vitro* and *in vivo*.

Conclusions. In view of the inhibitory effect of penicillin and terramycin on *C. perfringens* in the intestinal tract of turkeys and of terramycin in pigs, it appears possible that these antibiotics promote growth by preventing enterotoxemia. This possibility is supported by data gathered by Merchant(16) which show that enterotoxemia in sheep is caused by *C. perfringens.*

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Effect of Niacin and Tryptophan in Counteracting Toxicity of Crystalline Borrelidin for Rat.*† (18376)

J. M. COOPERMAN, S. H. RUBIN, AND B. TABENKIN.

From the Departments of Nutrition and Microbiology, Hoffman-La Roche, Inc., Nutley, N. J.

The production of pellagra-like signs in animals fed toxic materials has been described by several investigators. Stockman and Johnston(1) reported the isolation of an organic acid from corn which produced coma and death in monkeys, rabbits and guinea pigs. No attempt was made to counteract the effects with niacin. Woolley(2) concentrated a nitrogenous base from corn meal which, on addition to a synthetic ration, inhibited the growth of mice. Indolacetic acid was claimed to inhibit growth of rats(3), but the experiments were not confirmed(4). It was shown that adenine at high levels produced the symptoms of black tongue in dogs (5), but no attempt was made to counteract this effect with niacin. Krehl *et al.*(6) demonstrated the role of corn in the production of niacin deficiency in rats.

Berger, Jampolsky and Goldberg(7) have reported the isolation of a toxic crystalline antibiotic, borrelidin, from the culture broth

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* A preliminary report was presented before the Fed. Am. Soc. for Exp. Biol., Apr. 1950, Atlantic City.

† Roche Publication No. 221.

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TABLE I. Basal Ration.

	%
Casein	20
Sucrose	71
Salts IV*	4
Cod liver oil	3
Corn oil	2
	γ/day
Thiamine	200
Riboflavin	200
Pyridoxin	200
Calcium pantothenate	400
p-Aminobenzoic acid	1000
i-Inositol	1000
Choline	10000
Folic acid	10
Biotin	10
Vit. K	10

* Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, 1935, v109, 657.

of a species of streptomyces. Concentrates with similar *in vivo* and *in vitro* activities were found in corn steep. The experiments described in this paper were designed to test the effectiveness of niacin and tryptophan in overcoming the toxicity of crystalline borrelidin for rats.

Experimental. Sprague-Dawley rats weighing 40 to 50 g were housed in individual cages. The composition of the basal ration which was fed *ad libitum* is shown in Table I. The vitamin supplement was given by cup in sugar solutions 3 times a week and was promptly taken by the rats.

In the first experiment, 5 groups of rats were used to determine the effectiveness of niacin in counteracting borrelidin toxicity, and each received the basal ration with additional supplements. The first group, the controls, received an addition of 1 mg of niacin in the vitamin supplement. Subsequent experiments showed that niacin did not improve the growth of rats receiving the basal ration. The second group received a dose of 10 μg borrelidin per day. The borrelidin solutions were given with a syringe and blunted needle which was introduced into the rats' stomachs since it was found that the animals refused to accept borrelidin mixed with the vitamin supplement. The other 4 groups received 10 or 20 μg borrelidin with or without 1 mg niacin in the vitamin supplement as outlined in Table II. The results (Table II) indicate that niacin can partially overcome the toxicity of borrelidin when given under the conditions described above. Statistical analysis of the differences between Groups 2 and 3 showed these differences to be significant at the 1% level. In the group receiving 10 μg borrelidin per day, 2 rats succumbed during the first 2 weeks, whereas all survived in the group receiving 10 μg borrelidin plus 1 mg niacin. Nine out of 10 rats receiving 20 μg borrelidin per day died during the first 2 weeks compared to 4 out of 9 in the group receiving 1 mg niacin in addition to 20 μg borrelidin per day. It is interesting to note that borrelidin had its greatest effect during the first 2 weeks of the experiment.

In another experiment groups of rats were given 2 μg borrelidin per day. In this case, the controls gained an average of 33 g per week for the 4 week period compared to 31 g for the rats receiving 2 μg borrelidin per day and 34 g for the rats receiving 2 μg borrelidin plus 1 mg niacin per day. These differences were not significant.

Since it has been shown that tryptophan can replace the requirement for niacin in the rat(8), it was decided to determine the effectiveness of tryptophan in overcoming the toxicity of borrelidin. In these experiments, the borrelidin was added directly to the basal ration, as were the niacin and tryptophan supplements. The basal ration and vitamin supplement were identical to those employed in the first experiment. Eight groups of rats were used. Group 1 received the same ration as the controls in the first experiment. The other 7 groups received the basal ration containing 1 or 2 mg borrelidin per kilogram with or without niacin or tryptophan as outlined in Table III.

In this experiment all rats survived the 4 week test period. The results are shown in Table III. As in the first experiment the toxicity manifested itself to the greater degree during the first 2 weeks of the experiment. Although the differences between Groups 2 and 3 and between 2 and 4 were of a smaller degree for the 4 week period, statistical analy-

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TABLE II. Effect of Oral Administration of Borrelidin.

Group	No. rats	Daily supplement μg Borrelidin	Avg gain in wt, g 1st wk	2nd wk	3rd wk	4th wk	Total 4 wk	Per wk, 4 wk period
1	10	Controls	20	43	30	29	122	31
2	10	10	7.5	4(8)*	14(8)*	19(8)*	44.5(8)*	11(8)*
3	10	10 + 1 mg Niacin	12	17	28	29	86	22
4	10	20	—3	10(1)*	9(1)*	13(1)*	36(1)*	9(1)*
5	9	20 + 1 mg Niacin	7	11.5(5)*	19(5)*	22(5)*	65(5)*	16(5)*

* No. of rats in parentheses.

TABLE III. Effect of Feeding Borrelidin Mixed with the Ration.

Group	No. rats	Supplement per kilo ration, mg Borrelidin	Avg gain in wt, g 1st wk	2nd wk	3rd wk	4th wk	Total 4 wk	Per wk, 4 wk period
1	10	Controls	24	40	37	40	141	35
2	10	1	2	12	20	22	56	14
3	10	1 + 500 mg Niacin	5	24	25	26	80	20
4	10	1 + 5 g dl-Tryptophan	4	17	25	26	72	18
5	5	2	—6	3	3	7	7	2
6	5	2 + 1 g Niacin	—3	6	20	13	36	9
7	5	2 + 5 g dl-Tryptophan	—4	6	19	19	40	10
8	5	2 + 10 g Niacin	—5	8	22	19	44	11

sis revealed them to be significant at the 5% level. In the groups receiving 2 mg of borrelidin per kilogram ration, little growth occurred during the first 2 weeks. However, during the last 2 weeks, the rats receiving niacin and dl-tryptophan grew at a more rapid rate than those receiving borrelidin alone. Under these conditions the niacin and tryptophan effects were more marked.

Discussion. Although several authors have claimed to produce the effects of niacin deficiency in rats with toxic materials, they have either used non-crystalline material or have not reversed the effects with niacin. The experiments described above represent the first time that the toxicity of a crystalline, naturally occurring substance has been counteracted by niacin in the rat. It should be emphasized that complete protection was not provided by either niacin or tryptophan.

Conclusion. When crystalline borrelidin is given orally to rats at a level of 10 μg per day, poor growth results. This inhibition of growth can be partially overcome by supplementation with niacin. Two μg of borrelidin per day given orally is without effect upon the growth of rats whereas 20 μg per day results in a high mortality rate. Poor growth is also observed in rats receiving a basal ration containing levels of 1 and 2 mg borrelidin per kilo. The addition of niacin or tryptophan results in significantly better growth.

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Hypoglycemia Produced by Purified Anterior Pituitary Growth Hormone and Its Relationship to the Pancreas.*† (18377)

M. Kurtz,‡ R. C. de Bodo, S. P. Kiang, and A. Ancowitz.§

From the Department of Pharmacology, New York University, College of Medicine, New York City.

In previous publications(1-3) we demonstrated that purified anterior pituitary growth hormone exerts a potent anti-insulin action in hypophysectomized dogs. Within 18 hours of the first injection of growth hormone the exaggerated hypoglycemic response to insulin was diminished, and upon continued administration of the hormone the hypersensitivity to insulin was completely abolished. In addition, a concomitant diabetic state was produced.

Under certain circumstances, however, the administration of growth hormone was followed by a marked hypoglycemic response. A similar effect has been described by several investigators(4,5). This report is concerned with this hypoglycemia and its relationship to the pancreas.

Methods. This study was carried out on hypophysectomized dogs‖¶ and on acutely depancreatized, but otherwise intact, dogs. A part of the duodenum was resected in conjunction with the pancreatectomy, thereby assuring complete removal of the islet tissue and also minimizing liberation of insulin due to excess manipulation of the gland. The pancreatectomies and the succeeding 6 to 7 hour experiments were done under sodium pentobarbital‖ anesthesia. The majority of the dogs maintained a blood pressure of approximately 100 mm of Hg. In none of the dogs did the pressure fall below 50 mm of Hg. The hypophysectomized dogs used were all maximally sensitive to the hypoglycemic action of insulin(6-7). The insulin sensitivity tests were performed on trained unanesthetized dogs as described in an earlier publication(6); insulin (Lilly**) 0.025 unit/kg being used in all tests. Purified anterior pituitary growth hormone, Armour Lot No. 22KR1,†† was used in this study. Growth hormone of the same lot number was found to exert the anti-insulin action discussed above. In the experiments performed on the hypophysectomized dogs the dose of growth hormone varied from 0.3 to 1.0 mg/kg, administered intravenously. The dose of growth hormone used in the experiments on the acutely depancreatized dogs was 7.5 mg/kg, also given

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† This work was presented before the Amer. Physiological Society at Columbus, O. Sept. 1950. Abstract in the *Amer. Journal of Physiology,* 1950, v163, 727.

‡ Dazian Foundation Fellow.

§ Present address: Halloran Veteran Hospital, Staten Island, N. Y.

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‖ We would like to thank Dr. R. K. Richards of Abbott Laboratories for his generous supply of Sodium Thiopental (Pentothal) and Sodium Pentobarbital (Nembutal). The hypophysectomies were carried out under morphine-pentothal anesthesia.

¶ Topical Thrombin, which was most helpful in controlling hemorrhage during the operation, was kindly supplied by Dr. A. C. Bratton, Jr., of Parke, Davis and Co.

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†† We are greatly indebted to Drs. E. E. Hays and I. M. Bunding of Armour and Co. for their generous supply of Growth Hormone.

TABLE I. Effect of Growth Hormone (GH) and of Insulin (INS) on Blood Sugar of Hypophysectomized Dogs in the Post-Absorptive State.

(A) Time min.	Dog Hy8 Blood sugar in mg %			(B) Time min.	Dog Hy11 Blood sugar in mg %			
0	67	63	60	0	67	63	66	71
						GH†		
	INS* .025 μ/k	INS* .025 μ/k	GH† .5 mg/k		INS* .025 μ/k	1st day .6 mg/k	2nd day .6 mg/k	3rd day .6 mg/k
10	49	37	53	10	52	68	67	73
20	30	25	35	20	29	66	64	73
30	33	31	35	30	23	62	62	73
40	32	32	32	40	27	60	61	71
50	32	30	32	50	28	60	62	71
60	32	32	36	60	30	51	64	68
75	33	33	33	75	30	57	60	71
90	37	43	34	90		47	59	71
120	38	45	36	105	42			
150	37	53	34	120	48	38	54	63
180	42	55	35	150	48	40	57	71
210	48	63	34	180	54	38	57	70
240	56	63	34	210	57	43	62	66
				240	64	44	63	66
				420		70		

* Insulin intrav. at 0 time.
† Growth hormone intrav. at 0 time.

intravenously. Blood sugar determinations were done on venous blood samples, at frequent intervals, during the experiments.

Results. I. The results of representative experiments are recorded in Table I. When growth hormone was administered intravenously, for the first time, to 17 to 18 hours fasted hypophysectomized dogs a marked fall in blood sugar occurred. The onset and degree of this hypoglycemia may either be similar to that seen following the administration of insulin (Table I-A), or the onset may be delayed and the degree not as marked as after insulin (Table I-B). The duration of the hypoglycemia after growth hormone was longer than that seen after insulin in all the experiments. When a series of daily administrations of growth hormone were given (all intravenously and in the post-absorptive state), the hypoglycemic response appeared only after the initial injection (Table I-B). The progressively diminishing hypoglycemic response to the growth hormone injections was probably due, at least in part, to the development of the anti-insulin action brought on by growth hormone.

II. The growth hormone induced hypoglycemia has been attributed to an increased output of insulin, *i.e.* growth hormone exerts a pancreatotropic action. In an attempt to further elucidate the role of the islets of Langerhans in this hypoglycemia, the effect of growth hormone on the blood sugar of acutely pancreatectomized dogs was studied. Fig. 1 shows the blood sugar changes in 5 dogs following pancreatectomy. In 2 dogs the pre-operative blood sugar levels were higher than those generally observed. These 2 dogs were untrained and the high blood sugar values were probably due to increased adrenaline output. After removal of the pancreas the blood sugar steadily rose to hyperglycemic levels in each animal. Two of the dogs showed a slight and short lasting decrease in blood sugar 2½ and 3½ hours respectively after pancreatectomy. The blood sugar at the end of 6 to 7 hours varied between 152 and 509 mg %.

Several dogs were subjected to sham operations during which a portion of the jejunum was resected. No significant change in blood sugar occurred after the sham procedures. Thus, anesthesia, abdominal manipulation, and adrenaline secretion can be dismissed as factors responsible for the rise in blood sugar in the acutely depancreatized animals used in

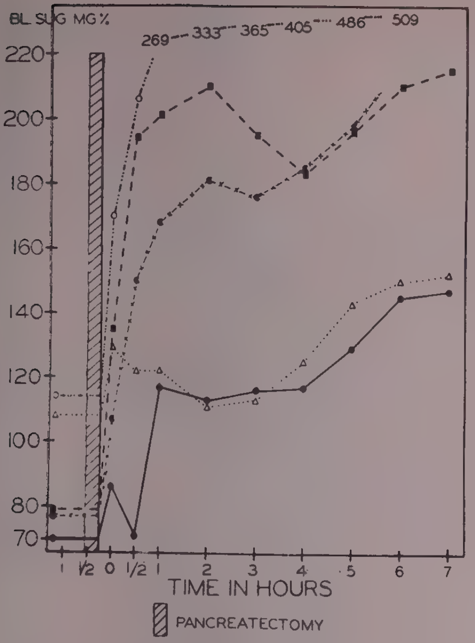

Fig. 1.

Blood sugar curves of 5 dogs during the 7 hours following pancreatectomy.

this study.

Fig. 2 shows the results following growth hormone administration to acutely pancreatectomized dogs. The pre-injection blood sugar levels were significantly above the pre-operative levels in 4 of the 6 dogs. Growth hormone was administered intravenously 38 to 48 minutes after removal of the pancreas. In every case a significant fall was apparent within 55 to 85 minutes of the injection of hormone. In 4 of the 6 dogs the blood sugar fell to levels as low as 35 to 54 mg % 3 to 5 hours after growth hormone. In the 2 instances in which the blood sugars were 158 and 202 mg % prior to the growth hormone injection, a significant decrease in blood sugar level occurred, but hypoglycemic levels were not reached. Toward the end of each experiment the blood sugar started to rise but in no case was the final blood sugar above the pre-growth hormone injection level; in fact in 5 of the 6 dogs it was below.

Discussion. It is evident from the data presented that purified anterior pituitary growth hormone is capable of producing a marked hypoglycemia in acutely depancreatized animals. The hypoglycemia observed in these animals must of necessity have been of extra-pancreatic origin. However, our experiments do not entirely exclude the possibility that in the intact animal the growth hormone induced hypoglycemia is due to some degree to a pancreatotropic action, *i.e.* a stimulation of insulin output from the islet cells.

Another aspect to be considered is that the acutely depancreatized animal is very probably not completely free of insulin. Therefore, the possibility exists that the presence of insulin is a necessary factor for the development of the growth hormone induced hypoglycemia.

Summary and conclusions. The administration of growth hormone to hypophysectomized dogs in the post-absorptive state results in a marked fall in blood sugar. This marked hypoglycemic response is seen only after the first of a series of injections of the hormone. The hypoglycemic effect of growth hormone also occurs in acutely depancreatized

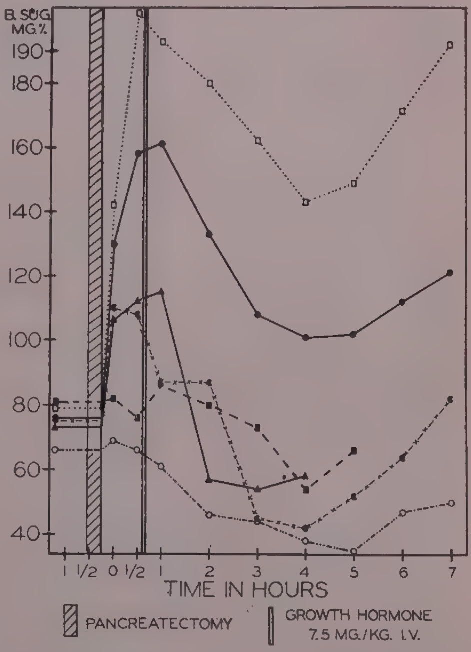

Fig. 2.

Effect of growth hormone on the blood sugar of 6 acutely depancreatized dogs.

dogs. It is concluded that the presence of the pancreas is not essential for the aforementioned action of growth hormone. However, it remains to be established whether insulin is a necessary factor for the growth hormone induced hypoglycemia.

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Effect of Pantothenic Acid Deficiency upon Adrenal Cortex, Thymus, Spleen, and Circulating Lymphocytes in Mice.* (18378)

R. M. Melampy, D. W. Cheng, and L. C. Northrop.

From the Department of Zoology and Entomology, Iowa State College, Ames, Ia.

During the course of an investigation on the distribution of pantothenic acid in the tissues of adult male mice of the CF No. 1 strain, it was possible to make observations on the effect of a deficiency of this dietary requirement on the white blood cell count as well as structural changes in the adrenal cortex, spleen, and thymus(1). Such a study is desirable because of the established functional interrelationship between the secretory activity of the adrenal cortex, lymphoid tissue, and alterations in the circulating lymphocytes (2).

Lippincott and Morris made histopathological studies on young and adult C3H strain mice maintained on a diet deficient in pantothenic acid until death intervened in 6 to 8 weeks and reported a hyperkeratotic, atrophic, and desquamative dermatosis as well as myelin degeneration in the sciatic nerves and in the spinal cord. These investigators also stated that other organs, including the adrenals, were essentially normal in the deficient animals(3).

Experimental. Five mature mice, approximately 3 months of age, were fed a pantothenic-acid deficient ration No. 80 as described in detail by Morris and Lippincott(4). Four control animals of the same age received the same ration except that it was supplemented with d-calcium pantothenate at the rate of 30 mg per kg. All animals were weighed every 3 days throughout the experimental period of 7 weeks. Total white cell counts and blood smears, stained with Wright's stain, were made at weekly intervals. Tail-vein blood was used. For a histochemical study of the effect of pantothenic acid deficiency on the adrenal gland, thymus, and spleen, a second group of 22 mature male mice was used. Seven control animals were placed on pantothenate-containing ration and 15 on pantothenate-deficient ration. Individuals were killed at various intervals during an experimental period of 7 weeks to furnish tissues. In addition, 6 male mice were placed on a pantothenate-containing ration for 2 weeks, after which they were allowed to starve. The average duration of life of these animals was 6.3 days. One adrenal of each pair was prepared for frozen sections by fixation in 10% neutral formalin while the other was prepared for paraffin sections by fixation in Zenker-formol followed by postchromation in 3% potassium dichromate for 3 days. Thymus gland was also fixed for frozen and paraffin sections. Spleen was fixed in Zenker-formol for routine examination. Frozen sections were cut at 10 μ and paraffin sections at 6μ. Lipids were characterized in frozen sections by Sudan IV, Sudan Black B, Oil Red O, Schiff's reagent, and 2-4 dinitrophenylhydrazine(5). Heidenhain's iron alum

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* Industrial Science Research Institute—Project 401.

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TABLE I. Effect of Pantothenic Acid Deficiency in Adult Male Mice on Body Wt, Total Leucocyte and Lymphocyte Counts.

	Avg body wt in g		Total leucocyte, thousand per cmm			Total lymphocyte, thousand per cmm		
Wk	Control	Deficient	Control	Deficient	Diff.	Control	Deficient	Diff.
0	25 ± 1*	25 ± 1	12181	12162	19	10584	10499	85
1	26 ± 1	25 ± 1						
2	26 ± 1	22 ± 1	13275	8675	4600†	11140	6794	4346‡
3	26 ± 1	22 ± 2	12581	10650	1931	10595	8455	2140
4	27 ± 1	21 ± 1	10025	6750	3275	8246	4186	4060†
5	26 ± 1	20 ± 1	14662	8750	5912	12490	4622	7868‡
6	27 ± 2	20 ± 1	14750	18138	3388	11450	8852	2598†
7	28 ± 1	20 ± 1	8688	18100	9412	6665	13097	6432

* Mean and stand. deviation $\sqrt{\frac{\Sigma d^2}{n-1}}$.

† Significant at P = .05.
‡ Significant at P = .01.

haematoxylin and eosin were used in the staining of tissues for routine study. Birefringence was determined on a polarizing microscope. Control sections for this method and the Schiff reaction were first extracted with acetone.

Results and discussion. Data relative to body weight changes and hematological findings are presented in Table I. It is to be noted that the average body weights of the animals on the pantothenate-free ration decreased, whereas the controls gained. In comparison with the controls the experimental animals developed a leucopenia, after which a significant leucocytosis appeared which was maintained until the seventh week. Test for significance was made according to Snedecor(6). These data also show a significant lymphopenia for 6 weeks followed by an insignificant lymphocytosis. Furthermore, an insignificant neutrophilia was observed throughout the deficiency period. It is suggested that the leucopenia observed for the first five weeks in the deficient animals was due to lymphopenia and the leucocytosis which occurred following the fifth week was probably the result of the neutrophilia. However, by the seventh week the leucocytosis may be attributed to the combined effect of the neutrophilia and lymphocytosis.

By applying the histochemical methods previously described, it was possible to demonstrate that the lipids of the adrenal cortex of control mice showed reactions indicating the presence of ketosteroids. They gave a Schiff plasmal reaction, formed a yellow phenylhydrazone after the application of phenylhydrazine and revealed birefringence on the polarizing microscope. However, the adrenal cortex of the deficient animal showed a gradual, progressive depletion of sudanophilic and ketosteroid material from the zona fasciculata when compared to controls. This decrease was only slight at the end of the second week and was more pronounced by the end of the sixth week of the deficiency as shown in Fig. 1, 2, and 3. The zona glomerulosa remained unchanged.

Furthermore, by means of areal analysis of paraffin sections it was possible to demonstrate a gradual hypertrophy of the adrenal cortex during pantothenic acid deficiency. By the seventh week of the deficiency the adrenal cortex appeared to show signs of exhaustion of steroid material as evidenced by the reduction in birefringent material, particularly in the zona fasciculata. This probably is a contributing factor in the development of the lymphocytosis occurring at this time as shown in Table I. This cortical hypertrophy suggests an increased secretion of cortical hormone and its lipid depletion indicates an output below the physiological requirements of the organism. Deane and Greep have shown that the zona fasciculata secretes corticosterone-like hormones which govern gluconeogenesis whereas the zona glomerulosa is the site of hormones influencing electrolyte bal-

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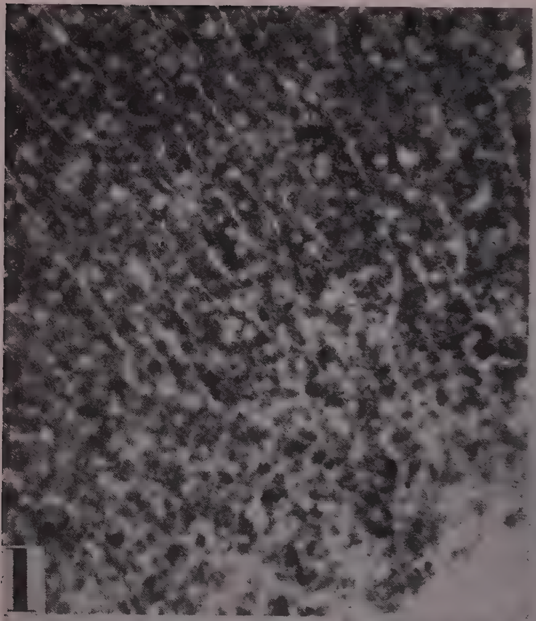

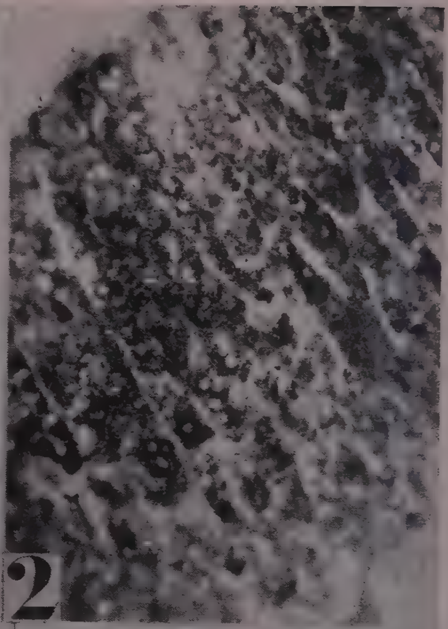

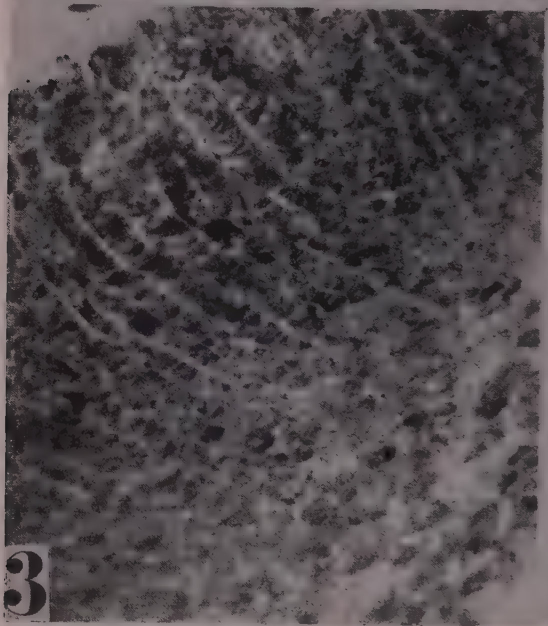

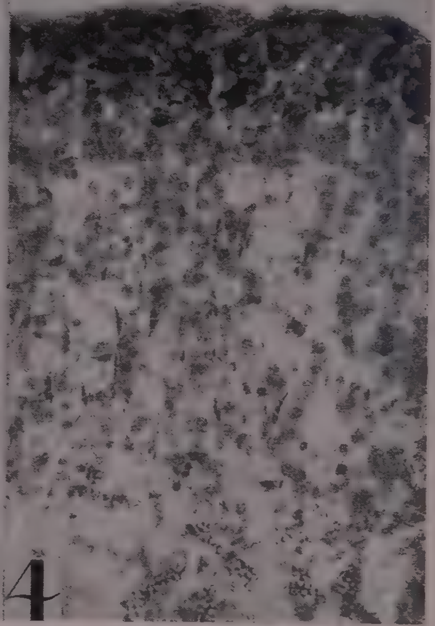

FIG. 1. Adrenal cortex from control animal stained with sudan black B and Mayer's carmalum. ×250.

FIG. 2. Adrenal cortex from animal on pantothenate-deficient ration 2 wk showing reduction of lipid in zona fasciculata. ×250.

FIG. 3. Adrenal cortex from animal on pantothenate-deficient ration 6 wk showing marked reduction of lipid from inner fasciculata. ×250.

FIG. 4. Adrenal cortex from starved animal showing total disappearance of lipid from zona fasciculata and presence of sudanophilic lipid in zona glomerulosa. ×250.

ance(7). There was no evidence of cortical necrosis or hemorrhage in the deficient mouse as has been reported for the rat. Furthermore, the changes occurring were more gradual in their appearance than in the case of the rat(8). Starvation caused complete exhaustion of sudanophilic and steroid materials from the zona fasciculata as shown in Fig. 4. In contrast, the zona glomerulosa of these animals retained some sudanophilic lipids but was practically depleted of ketosteroids. This would suggest that the physiological demands upon the adrenal cortex were much greater in starvation than in pantothenic acid deficiency.

The spleen weight of mice increased during the deficiency when compared with the controls but the amount of white pulp decreased significantly by the end of the sixth week as shown by areal analysis of sections. Histological examinations of spleen tissue from deficient mice indicated the following: (1) germ centers in the white pulp were rarely visible, (2) there were relatively few lymphocytes, and (3) lymphocytolysis was apparent as shown by the pycnotic nuclei. Furthermore, there was no indication of cell division. These changes may be correlated with the occurrence of the significant lymphopenia as shown in Table I. By the end of the seventh week the white pulp of the spleen was similar in appearance to that of the 6-week control in that the germ centers were active and the amount of white pulp had increased.

During pantothenic acid deficiency in the adult mouse, the thymus gland underwent involution and this was accompanied by an apparent increase in sudanophilic lipid in the interlobular and subcapsular connective tissues. By the seventh week there was a marked decrease in the number of lymphocytes in the thymus and as a result, the reticular network of the gland was much more conspicuous than in control animals. Dougherty and White have shown a direct relationship between the degree of involution of lymphoid tissue and the amount of corticosterone secreted(2).

Summary. Adult male mice placed on a pantothenate-deficient diet developed a lymphopenia followed by lymphocytosis when compared to control animals. The adrenal cortex showed hypertrophy and simultaneous, gradual depletion of lipid material, including ketosteroids, from the zona fasciculata. By the seventh week the zona fasciculata was nearly depleted of its hormone. In deficient mice, the thymus atrophied and there was a gradual enlargement of the spleen. Starvation caused a rapid depletion of the lipid, including the ketosteroids, from the zona fasciculata. The zona glomerulosa remained unchanged in pantothenic acid deficiency whereas in starvation it was depleted of steroid material but retained some sudanophilic lipids.

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"Spontaneous" Leukemia Developing in C3H Mice Following Inoculation, In Infancy, with AK-Leukemic Extracts, or AK-Embryos.* (18379)

Ludwik Gross.

From the Cancer Research Unit, Veterans Administration Hospital, Bronx, N. Y.

The purpose of this study was to determine whether "spontaneous" leukemia could be prompted to develop in middle aged mice, of a line known to be essentially free from this disease, by inoculating such animals, in their early infancy, with extracts prepared from mouse leukemia, or with cell suspensions prepared from embryos removed from the uterus of a mouse of a leukemic inbred line.

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Materials. Animals. It was necessary for this study to use animals having a family record essentially free from leukemia. Mice of the C3H line appeared to be suitable for this purpose because they rarely, if ever, developed spontaneous leukemia(1). In our colony of C3H mice we have observed thus far a total of 2,540 females and 2,000 males and we have seen only 3 cases of spontaneous leukemia among these mice. Because, however, females of the C3H line develop mammary carcinomas, mice of a foster nursed C3H subline have been used for this study. A colony of such mice has been raised in this laboratory from a litter of C3H mice that had been nursed by a C57 female. These mice have been designated in this study by the symbol C3H (f). Up to the time of this writing we have observed 745 females and 432 males of this line through 16 successive generations, and there has been thus far no case of spontaneous leukemia among these animals (1).

Leukemic extracts. Mice of the Ak line that developed leukemia either spontaneously, or as a result of inoculation with a transplanted strain of leukemia, were sacrificed by ether inhalation, and fragments of liver, spleen, and enlarged lymph nodes, as well as small pieces of a mesenteric tumor regularly found in such animals, were removed aseptically. The leukemic tissues were weighed, then placed in a sterile mortar, cut with small scissors, and ground thoroughly for 5 minutes, isotonic sodium chloride solution being added to obtain cell suspensions of 20% concentration. The suspensions were cleared from larger tissue pieces by passing them through a sterile voile cloth filter; the resulting cell suspension was used for inoculation of infant mice in Exp. No. 1126. In experiments 1100, 1101 and 1106, the leukemic cell suspensions, obtained in the manner described above, were centrifuged at 3,000 RPM for 15 minutes in a refrigerated PR-1 International Centrifuge at 0°C; the supernatant was removed carefully, placed in a new test tube, and again centrifugated for additional 15 minutes under identical conditions (3,000 RPM at 0°C). The second supernatant was then carefully removed with a tuberculin syringe, and used immediately for inoculation.

Embryo-cell suspension. A 4-month-old, healthy, pregnant female mouse No. 476 of the Ak inbred line was sacrificed, and 6 embryos were removed aseptically from the uteri. Over 70% of females of our colony of Ak mice developed spontaneously leukemia after they reached 7 months of age(1,2). The Ak female No. 476 was in good health at the time the embryos were removed from her uteri, because she was then only 4 months old. Her mother, Ak female No. 417 was also sacrificed, when in good health, when 5 months old, for experimental purposes. The grandmother, however, as well as great-grandmother, and several other ancestors on both her maternal and paternal sides had developed, and died from, spontaneous leukemia. The embryos thus obtained were cut with small scissors, and then ground thoroughly in a sterile mortar, isotonic sodium chloride solution being added to obtain a cell suspension of approximately 20% concentration. This cell suspension was then passed through a sterile voile cloth filter, tested for bacterial sterility, and used immediately for inoculation.

Experimental procedure. Females of the C3H (f) line were mated to their brothers in individual wooden cages; when they became pregnant, they have been watched at frequent intervals, and the birth of each litter was promptly recorded. In experiments reported in this study only litters less than 12 hours old were used for inoculation. Older suckling mice were also inoculated, but they will be reported separately(3). A tuberculin syringe, and a 27 gauge needle were used for the injection. The suckling mice were returned immediately after inoculation to their mothers. After they have reached 21 days of age, these mice were weaned, marked individually, and placed in separate cages. At the age of 2 to 3 months, the females were

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permitted to have one litter, having been mated to one of their respective brothers. After the litters had been weaned, these mice were separated according to sex, and were from then on kept under observation.

Results. Inoculation of newborn C3H (f) mice with leukemic cell suspensions. It was observed in our previous experiments that newborn mice of the C3H line are highly susceptible to inoculation with Ak leukemic cell suspensions. Of the 54 one-day-old infant mice inoculated, all developed acute leukemia in from 10 to 12 days(1). *Exp. 1126.* When, however, 5 one-day-old mice of the C3H line were inoculated subcutaneously with the Ak leukemic cell suspensions (0.1 cc each), only 4 of them developed acute leukemia within 12 days; the fifth infant mouse, a female, remained in good health; yet, after 7 months, this mouse developed generalized lymphatic leukemia. No leukemic infiltration was found under the skin, at the site of the initial subcutaneous inoculation.

Inoculation of newborn C3H (f) mice with Ak leukemic centrifugated extracts. Since the inoculation of newborn infant mice of the C3H line with Ak leukemic cell suspensions resulted in most instances in prompt development of acute leukemia, centrifugated leukemic extracts were used in experiments 1100, 1101, and 1106. These centrifugated leukemic extracts were injected subcutaneously, and/or intraperitoneally, into infant mice of the C3H (f) line. The inoculations were made within 12 hours after the birth of these animals. Of the 14 mice inoculated in 3 experiments, 7 developed generalized leukemia in from 8 to 11 months after the inoculation, as follows: *Exp. 1100.* Infant mice (1 female and 4 males) born on Jan. 9, 1950 to a C3H (f) female, were inoculated subcutaneously (each 0.1 cc) with Ak leukemic centrifugated extract, prepared from a transplanted Ak leukemia. Fourteen days later, a similar Ak leukemic centrifugated extract was freshly prepared from the same strain of transplanted leukemia, and the infant mice were reinoculated, this time intraperitoneally (0.1 cc each). These animals were weaned on February 3, 1950, and marked individually (female No. 22, males No. 293, No. 294, No. 295 and No. 296). All 5 mice remained in good health until October 1950. On October 13, 1950, 2 of these 5 mice, namely female No. 22, and male No. 295, were found to have generalized leukemia undistinguishable from "spontaneous" leukemia so frequently observed in middle aged mice of the Ak line. Aprroximately 2 months later, male No. 296 also developed generalized leukemia. None of these 3 leukemic mice had any local new growth at the site of the initial subcutaneous inoculation. *Exp. 1101.* A centrifugated extract was prepared from leukemic organs of a mouse of the Ak line that developed leukemia spontaneously. This extract was injected subcutaneously (0.1 cc each) into 4 infant mice (1 female and 3 males) of the C3H (f) line born less than 12 hours prior to inoculation on January 10, 1950. These infant mice were weaned on January 31, 1950 and marked individually (female No. 15 and males No. 286, No. 287 and No. 288). All 4 mice remained in good health until September, when male No. 287 developed generalized leukemia and died within a few days (September 7, 1950); there was no local infiltration at the site of the initial inoculation. On October 16, 1950, female No. 15 was found to have enlarged lymph nodes, and a few days later her spleen also became palpable. Blood count (from tail) revealed WBC 40,000 with 75% lymphocytes. The mouse was sacrificed, and a typical picture of generalized leukemia was found, with no evidence, however, of any local growth at the site of the initial subcutaneous inoculation. *Exp. 1106.* On January 16, 1950, a centrifugated leukemic extract was prepared in the usual manner from a transplanted Ak leukemia, and injected subcutaneously (0.1 cc each) into 8 infant mice of the C3H (f) line, less than 12 hours old. Of these 8 infant mice, 3 died within 2 weeks following inoculation. The 5 mice that survived, were weaned at 21 days of age, and given individual numbers (female No. 93, and males No. 63, No. 64, No. 65 and No. 66); they remained in good health until the first week in November, 1950; at that time male No. 63 developed generalized leukemia without any local growth at the site of the initial, subcutaneous inoculation; a few days later, male No. 65 also was found to have developed leukemia.

Results of inoculation of Ak-embryos cell suspensions into infant mice of the C3H (f) line. Newborn infant mice of the C3H (f) line were inoculated subcutaneously, and also intraperitoneally, with cell suspensions prepared from Ak embryos. Suckling infant mice varying in age from those a few hours, to those several days old, were used for the inoculations. In this report, however, only 2 experiments are reported in which the infant mice were inoculated when they were less than 12 hours old; all the other experiments, dealing with mice inoculated at a later age, will be reported separately(3), because the results of all these other experiments have been thus far negative. *Exp. 1154.* On February 24, 1950, a cell suspension prepared from Ak embryos was injected subcutaneously, and also intraperitoneally (0.1 cc each), into 6 infant mice of the C3H (f) line, less than 12 hours old. Only 2 of these 6 infant mice survived the inoculation; the remaining 4 died after a few days. Of the 2 mice that survived, one (male No. 341) developed generalized leukemia at 8½ months of age, with no sign of any local growth at the site of the initial subcutaneous inoculation. *Exp. 1155.* On the same day (February 24, 1950) the Ak-embryo-cell suspension was injected subcutaneously and intraperitoneally (0.1 cc each) into 6 infant mice, less than 12 hours old, of the C3H (f) inbred line. Of these, 1 died within a week, and another was killed accidentally at the age of 3 months. Of the 4 mice that survived (females No. 118, No. 119 and No. 120, and male No. 96), one female (No. 118) developed generalized leukemia at 8½ months of age; her blood count, taken from tail, revealed 260,000 WBC with 65% of lymphocytes; there was no evidence of any local growth at the site of the initial subcutaneous inoculation. At the same time, male No. 96 also developed generalized lymphatic leukemia; in this animal again there was no evidence of any local growth at the site of the subcutaneous inoculation. One month later, female No. 120 was also found to have developed generalized leukemia.

Cell-transplantation of leukemia that developed spontaneously in C3H (f) mice. In an effort to bring some clarification of the nature of leukemia that developed in mice of the C3H (f) line reported in Experiments 1100 and 1101 of this study, an attempt was made to transplant this leukemia, by cell-transfer, into adult mice of the same inbred line. Accordingly, cell suspensions of 20% concentration, were prepared from C3H (f) females No. 22, Exp. 1100, and No. 15, Exp. 1101, and inoculated (0.1 cc each) intraperitoneally into 8 adult mice of the C3H line; as a result, all inoculated animals developed acute leukemia within 2 weeks. The results of both experiments suggest that females No. 22 and No. 15 developed "spontaneous" leukemia, since in both instances leukemic cells prepared from the donor animals were readily transplantable to adult mice of the same inbred line. Had these 2 mice (females No. 15 and No. 22) developed a "transplantable" Ak leukemia, as a delayed result of an inoculation, in their infancy, of Ak leukemic cells, a subsequent attempt to transplant the leukemic cell suspension from either mouse No. 22, or No. 15 to adult mice of the same C3H (f) line would have most likely not succeeded, unless massive doses had been employed(1,3).

Discussion. Experiments reported in this paper suggest that "spontaneous" leukemia can be prompted to develop in middle aged mice of a line known to be essentially free from this disease, by inoculating such mice, not later than 12 hours after their birth, with leukemic extracts prepared from either spontaneous, or transplanted mouse leukemia, or, in some instances at least, with embryo-cell suspensions prepared from apparently healthy mouse-embryos of a leukemic inbred line.

One could, perhaps, assume that leukemia developing in such middle aged animals is not "spontaneous," but may be a delayed result of the implantation, in infancy, of some leukemic cells that might have been present in the centrifugated extracts. The development of a "transplanted" leukemia, however, would rather be expected to occur much more promptly, within 2 to 3 weeks, usually(1);

another reason for doubt would be the fact that following subcutaneous inoculation of the centrifugated leukemic extracts, generalized leukemia developed in these hosts after middle age, without, however, any local new growth at the site of the initial subcutaneous inoculation. Finally, the successful results of the transplantation of leukemic cells from our C3H (f) mice No. 22 and No. 15, into adult mice of the same inbred line, are consistent with the assumption that females No. 22 and No. 15 had developed "spontaneous" leukemia, although it must be stated that in some instances, at least, Ak leukemic cells may also reproduce acute leukemia when inoculated, in large doses, into adult mice of the C3H line(1,4).

The development of leukemia in 4 out of 6 middle aged mice, that had been inoculated, within 12 hours after birth, with embryo-cell suspensions prepared from mouse embryos of a leukemic inbred line, is also quite interesting. No possibility of a leukemic cell implantation needs in these instances to be considered, since the cell suspension was in this experiment prepared from perfectly healthy embryos that had been removed aseptically from a healthy, young female mouse of a leukemic inbred line. The donor's mother had also been sacrificed in good health, but her grandmother, as well as great-grandmother, etc., died from leukemia. One could, perhaps, wonder whether the mice reported in this study developed leukemia by some obscure process of "mutation." One has, however, to consider the fact that in this particular inbred line leukemia develops in untreated animals only exceptionally, if at all. In our laboratory we have observed over 1100 mice of this foster nursed C3H subline, through 16 successive generations, without having found a single case of spontaneous leukemia among the untreated animals.

It would appear more logical to assume that these mice developed leukemia, in middle age, as a direct result of inoculation, in their early infancy, with a transmissible, leukemic agent. It would then be necessary to consider that this agent was present in the leukemic cells as well as in centrifugated leukemic extracts, and also in the Ak-embryo cell suspension; the leukemic agent was inoculated, into the newborn mice, in its inactive form; it remained inactive through the early adult life of its carrier-hosts; when the infected hosts, however, reached 8 to 11 months of age, the agent became, in some of them, at least, activated, causing the development of leukemia. Such a working hypothesis(5,6) may be new for mouse leukemia, but it has already been demonstrated to be true for chicken lymphomatosis(7,8). In principle at least, such a leukemia would be similar to mammary carcinoma which develops in middle aged female mice as a result of the infection of these animals, through mothers' milk(9), or by experimental inoculation, in their early infancy, with the mouse mammary carcinoma agent (10,11). The term "activated" would be, perhaps, more accurate than the term "spontaneous," since it is reasonable to assume that an activation of a hitherto latent agent, may be responsible for the "spontaneous" development of these neoplasms(5,6).

There are, however, important differences between the as yet hypothetical transmission of a mouse-leukemia agent, and the already established facts concerning the transmission of the mouse mammary carcinoma. Thus, the mouse mammary carcinoma agent is transmitted from mothers to their offspring through milk(9,10); in the case of mouse leukemia, however, it appears that the milk of nursing mothers is not responsible for the transmission of this disease from one generation to another(12-14); if such a transfer occurs at

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all, therefore, it would have to be explained by a direct transmission of the leukemic agent through the germinal cells, *i.e.* through the embryo, as in the case of chicken lymphomatosis(7,8). Another important point of difference between the agent of mammary carcinoma and that of mouse leukemia would refer to the time-limit of susceptibility of the hosts to experimental infection with these respective agents. When the experiments reported in this study were planned, we have anticipated that the susceptibility of mice of the C3H line to inoculation with the hypothetical leukemic agent may well be limited to the first days, or perhaps even weeks, of their lives; this assumption was based on certain theoretical considerations, mainly, however, on the fact that the susceptibility of mice to the experimental inoculation with the mammary carcinoma agent is for all practical purposes limited to their first 3 or at the utmost 4 weeks of life(10,15,16); similarly, the susceptibility of chickens to experimental inoculation with lymphomatosis has been found to be limited to their first few days of life(17). For these reasons, young, suckling infant mice were used for inoculations. Because, however, the older suckling mice are much more resistant to handling and to injections, the majority of those used for our experiments were 2 to 7 days old at the time of inoculation. Only very few newborn infant mice, less than 12 hours old, were used for inoculations with the leukemic extracts and, curiously, these were the ones that proved to be susceptible. Although more than 80 infant mice of the C3H (f) line were inoculated with leukemic extracts when they were more than 2 days old, they have all remained in good health up to the time of this writing(3). Similarly, only very few infant mice less than 12 hours old were used for inoculation with the Ak-embryo cell suspensions, and only some of those proved to be susceptible; when, however, older infant mice were inoculated, at ages varying from 2 to 7 days (25 females and 23 males)(3), none has developed leukemia up to the time of this writing, although more than one year has already passed since most of these experiments have been performed. Thus, the susceptibility of mice to experimental inoculation with the leukemic agent may well be limited to the very first hours of their lives.

Summary. 1. Seven of 14 C3H mice inoculated when less than 12 hours old with Ak leukemic extracts developed leukemia at 8 to 11 months of age. 2. Four of 6 C3H mice inoculated, when less than 12 hours old, with cell suspensions prepared from Ak embryos, developed leukemia at 8½ months of age. 3. No leukemia resulted when mice more than 12 hours old were used for inoculation. 4. These experiments suggest that spontaneous mouse leukemia may be caused by an agent, which is transmitted from one generation to another, like that of chicken lymphomatosis.

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Manganous Ion and Activation of Prothrombokinase.[*] (18380)

J. H. Milstone. (Introduced by H. S. N. Greene.)

From the Laboratory of Pathology, Yale University School of Medicine, New Haven, Conn.

At the time 3-stage analysis of blood coagulation was originally described, it was reported that the activation of prothrombokinase involves an autocatalytic or chain reaction(1). The simplest explanation seems to be that thrombokinase is an enzyme, capable of activating both prothrombin and prothrombokinase(2).

1. $\text{Prothrombokinase} \xrightarrow{\text{Thrombokinase}} \text{Thrombokinase}$
2. $\text{Prothrombin} \xrightarrow{\text{Thrombokinase}} \text{Thrombin}$
3. $\text{Fibrinogen} \xrightarrow{\text{Thrombin}} \text{Fibrin}$

In crude systems, calcium is important for the normal progress of the first 2 reactions. Subsequent results with the 3-stage procedure (3,4) have favored the view that the true activator of the prokinase is kinase, as indicated above, rather than thrombin or lipoid thromboplastin. Further usefulness of the 3-stage procedure is demonstrated by the results with manganous ion.

In unpublished experiments by Hellerman, Milstone and Carnes (mentioned by Hellerman(9), p. 479) it was found that Ni^{++}, Co^{++}, and Mn^{++} retarded production of thrombin in crude systems [cf. Deetjen(5) and others (6-8)]. At that time it was noted that Mn^{++} which inhibited only at relatively high concentrations, also had an acceleratory effect at low concentrations. Fig. 1 illustrates the results obtained by the 2-stage procedure and confirms the earlier unpublished findings. An outstanding effect was shortening of the latent period. The latent period is concerned with the activation of the prokinase which is contained in the crude prothrombin(2).

The effect of 0.0001 M Mn^{++} on the activation of crude prokinase is brought into bold relief by the 3-stage test of Fig. 2. In the presence of Mn^{++}, the peak kinase activity was reached in half the time required for the control. The decline of kinase activity was rapid, as it also was in the control. Subsequently, the activity of the control remained

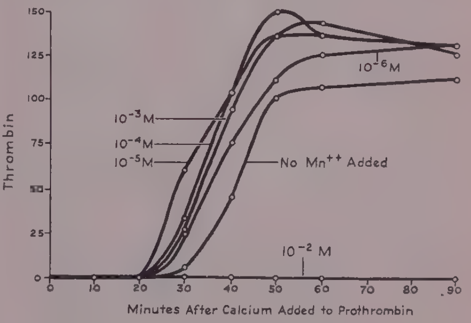

FIG. 1.

Two-stage experiment, showing that latent period in activation of crude prothrombin was shortened by addition of Mn^{++}. A stock 0.11 M sol. was prepared from Mallinckrodt analytical reagent, $MnCl_2 \cdot 4H_2O$; and 10-fold dilutions were made in buffer. The figures on the chart give approximate final molarities of added Mn^{++} in respective activation mixtures. Activation mixtures contained 0.1 ml heated crude prothrombin, 0.8 ml buffer, 0.1 M. $MnCl_2$ dilution and 0.1 ml $CaCl_2$ 0.0275 M. Mn^{++} was added 15 sec. before Ca^{++}. The tests were started at one-minute intervals and run in parallel, using same batches of reagents. Materials were prepared and tests conducted as previously described(3). The latent period was shortened by all tested concentration of Mn^{++}, except 10^{-2} M. In the presence of 10^{-2} M Mn^{++}, appearance of thrombin was completely suppressed during the test period.

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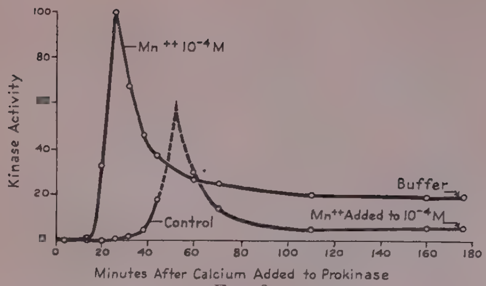

FIG. 2.

Three-stage experiment showing that Mn^{++} accelerated the activation of crude prokinase. Method and materials were essentially those described elsewhere(3). Mn^{++}, to make a final concentration of 10^{-4} M, was added to prokinase 15 sec. before Ca^{++}. At intervals, samples were assayed for kinase activity by determining amount of prothrombin converted to thrombin in 4 min. For example, at 26 min. (first peak on chart), a sample from prokinase mixture (stage 1) was added to prothrombin reagent (stage 2). This stage-2 mixture, sampled at 4 min., gave a clot in 30 sec., when tested on oxalated fibrinogen (stage 3). Test and prokinase control, with buffer instead of Mn^{++}, were run one minute apart. 174 min. after the start, Mn^{++} to make a final concentration of 10^{-4} M was added to control. This addition reduced the concentration of protein and calcium by 9%. At the same time, a similar 10/11 dilution of the test was made with buffer. These slight dilutions, as well as the uncontrolled 10/11 dilution of Mn^{++} in the test mixture, had no appreciable effect, as shown by the results. It is notable that the late addition of Mn^{++} to the control did not raise its kinase activity, as measured 2 min. after Mn^{++} was added.

below that of the test and was not then raised by addition of Mn^{++} to 0.0001 M. Thus the principal effect of Mn^{++} was exerted before or during the activation of prokinase, and could not be attributed to an enhancement of ripe kinase activity, as *measured by the conversion of prothrombin.* In other words, Mn^{++} did not appreciably accelerate the activation of prothrombin by kinase, under these conditions. This corroborates the impression gained from Fig. 1. There it is seen that, once conversion of prothrombin began, 10^{-3} to 10^{-6} M Mn^{++} exerted little effect on its rate, with the result that the rising portions of the curves were almost parallel.

Supplementary tests showed that 0.001 M Mn^{++} was more effective than 0.00001 M Mn^{++} in this 3-stage system. Calcium 0.0025 M, was present in all activation tests.

Discussion. The contrast between Figs. 1 and 2 emphasizes that the 3-stage test brings out details not readily discernible in 2-stage tests. Consequently, the new test serves not only to localize certain effects, but also to raise new questions. Mn^{++} caused much acceleration of the activation of prokinase and little, if any, of the conversion of prothrombin. Even if the materials were pure, these results would not necessarily militate against the hypothesis that the same enzyme catalyzes both activations. Calcium salts hasten the activation of trypsinogen, but not of chymotrypsinogen, although both conversions are catalyzed by trypsin(10). Since the materials are not pure, it is not known whether Mn^{++} brings about the acceleration by reacting with some impurity in the prokinase; nor is it known whether Mn^{++} would accelerate conversion of prothrombin under other circumstances.

The rapid loss of kinase activity promises to offer difficulties as more attention is given to the activation and assay of prokinase. It is difficult to judge the maximum activity when the peak is sharp, or when it falls between 2 points, as illustrated by the control curve of Fig. 2. Even the true peak probably represents the difference between the total activity developed and that portion of it which has already been lost.

In the present tests, the rise of kinase activity was probably much slower than occurs physiologically; and this may also be true for its fall. Relative to its rise, the decline of kinase activity was so precipitous as to suggest that it could help, *in vivo,* to keep the clotting process within physiologic bounds (11). The *in vitro* effect is possibly related to the losses of kinase or thromboplastic activity reported by others(12-14). It may represent a decrease of kinase, *per se,* or a change in some factor which conditions kinase activity.

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Summary. Manganous ion, 0.0001 M, hastened activation of crude prothrombokinase in a 3-stage analysis of blood coagulation. It did not prevent the subsequent steep decline of thrombokinase activity. Rapid quenching of thrombokinase activity might help in the physiologic control of blood clotting.

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Relation of Hair Proliferation to Damage Induced in the Mouse Skin. (18381)

HERMAN B. CHASE* AND WILLIAM MONTAGNA.†
(Introduced by Paul F. Fenton.)

From the Arnold Biological Laboratory, Brown University, Providence, R. I.

Physiological conditions prevailing in the skin at the time of experimental treatment with chemical or physical agents may be expected to influence the nature and degree of the response. A condition that may be readily observed and controlled is the stage of development of the hair. In the developmental cycle of a hair follicle, the anagen stage is the phase of active growth and development (17 days). In a more detailed analysis of the anagen phase, Chase *et al.*(1) have observed 6 clearly defined substages. The subsequent catagen stage is the phase of growth cessation, club hair formation, and setting aside of the dormant hair germ for the next hair generation (2 days). The telogen stage is the resting phase(2). The degree of injury which can be inflicted upon skin appears to depend not only upon the types of treatment but also upon the stage of development of the existing hair follicles. X-radiation with 300 r to 1000 r during anagen stages results in cessation of development in the follicle and the loss of any growing hair. During catagen and telogen stages, such x-ray doses cause no loss of hair, the club hairs remaining apparently undisturbed(3). In addition, x-radiation has also a profound effect upon hair pigment. Pigment loss for the ensuing hair generations is greatest in follicles x-rayed in catagen or telogen(3-5). In contrast to the effects of x-ray on the hair, single or repeated applications by painting of irritants such as K_2PO_4, ether, benzene(6), or methylcholanthrene-in-benzene have no effect on hairs in anagen but cause loss of hairs in the telogen stage. Whereas such doses of x-radiation have only a slight effect upon the pigment cells in the epidermis, upon the integrity of sebaceous glands, and upon hyperplasia of the epidermis—a single painting of 0.6% methylcholanthrene-in-benzene results in increased melanization in dendritic cells of the epidermis, loss of sebaceous glands(7-9), and hyperplasia of the epidermis. The painting, however, has no effect upon the growing hairs. After the loss of sebaceous glands by treatment with methylcholanthrene, a complete redifferentiation of the glands from external sheath cells occurs if and when there is active hair development in the follicles(9).

Methods. Experiments were carried out to

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determine the extent of injury in the epidermis and in the cutaneous appendages following application of methylcholanthrene alone and in combination with exposure to 1000 r of x-rays. In order to ascertain whether the stage of the hair follicles had any significant influence on the extent or type of injury induced by these agents, the right half of the dorsum of mice whose hair follicles were in the telogen (resting) stage, was plucked, while the left side was merely clipped. Plucking of club hairs initiates the growth of the next hair generation in that area whereas careful clipping or shaving does not(3). Eighteen mice (13 males and 5 females, 3 to 6 months of age) of the inbred C57 Black strain were used. In the first experiment the carcinogenic agent was applied after a period of 6 days when the clipped area was still in the telogen stage, and the plucked area was in substage anagen IV (an active phase of hair development in which the bulb is at maximal mitotic activity although the tip of the hair shaft has not yet emerged through the skin surface)(1). At this time, then, the whole dorsum was painted with 0.6% methylcholanthrene-in-benzene while only the posterior one-half of the dorsum received in addition an x-ray dose of 1000 r (100 kv, 10 ma, 17.4 cm, 389 r/min, air dose). Thus, in the same animal 4 skin areas were obtained in which conditions were different. To simplify matters these areas will be referred to as A, B, C and D (A, anterior left half with club hairs clipped, and treated with methylcholanthrene; B, anterior right half with club hairs previously plucked, and now in substage anagen IV, treated with methylcholanthrene; C, posterior left half, like A but, in addition, irradiated with 1000 r; D, posterior right half, like B, but also irradiated).

In a second experiment all of the skin areas treated were in the telogen stage. The left half of the dorsum was simply clipped while the right half was plucked at the time of treatment with methylcholanthrene and x-ray. The manipulations of this experiment were similar to the first experiment, with the exception of the 6-day interval between the time of plucking and the treatments.

Observations. Daily gross observations in the first experiment show in area A, a progressive keratosis and epilation of the stumps of clipped hairs, which persist a variable period of time up to 17 days following treatment when the experiment was terminated. In many instances, by the end of the experiment, the hair follicles are reactivated and growth proceeds apparently normally. In area B there is a moderate keratosis but no inhibition of hair growth. In area C there is keratosis, epilation, and subsequent ulceration which in some cases continues to the termination of the experiment. Area D shows some keratosis, a retardation of hair growth, and occasionally some ulceration.

In the second experiment (treated at the time of plucking and clipping) areas A and B are comparable to A of the first experiment, *i.e.* telogen follicles. Area B shows a more progressive keratosis and occasional ulcerations. In some cases, A as well as B has entered the anagen stage by the time of termination of the experiment. Areas C and D become keratotic and ulcerated. Although these two regions are comparable (they are also comparable to C of the first experiment), D, which has been plucked rather than clipped, in most cases shows a more advanced state of injury.

Histological sections of biopsies taken at 1, 4, 7, 10, 13 and 17 days after treatment substantiate the gross observations. A more extensive description of the cytological effects will be published in a separate paper. Area A in the first and in the second experiment, shows hyperkeratosis and hyperplasia of the epidermis accompanied by hypertrophy of the nucleus and cytoplasm in both surface epidermis and in cells of the hair follicles. These conditions are progressive for about 10 days and then recede. In some cases the condition is alleviated by the end of the experiment. Amelioration is always accompanied by reactivation of the hair follicles.

Area B in the first experiment shows only a moderate hyperplasia and keratosis up to the 4th day. A normal condition, including a restoration of the sebaceous glands is observed by the 10th day. In the second experiment, the epidermis of area B shows massive amounts of hyperplasia with hyperkeratosis and parakeratosis. There are numerous giant cells with nuclear aberrations. Moderate acanthosis is often found. These changes are progressive until the 10th or 13th day at which time there is some repair of the injury which is accompanied by a restoration of hair growth activity.

Areas C, in the first and second experiments, are alike. Massive progressive hyperplasia and hyperkeratosis of the epidermis and hair follicles occurs at first. Gigantic epidermal cells having either giant nuclei or clusters of nuclei are numerous throughout the duration of the observations. In sites of ulcerations single flattened giant cells may often be seen extending over the granular tissue of the dermis. In other places large areas of hyperplastic epidermis are underlain by invading epidermis. In still other areas, nothing remains of the epidermis except a few cords of giant cells extending into the dermis. Damage here in area C is severe and at the termination of the experiments only rarely did we encounter even slight amelioration or repair.

Area D in the first experiment, with follicles in anagen IV at the time of treatment, shows some hyperkeratosis and hyperplasia. This, however, is not severe. There is, in addition a cessation of growth of the hair follicles which lasts a variable interval of time. In all cases, however, the skin appears to be normal by the end of the experiment. In the second experiment, however, area D, with follicles in telogen at the time of treatment but the club hairs plucked instead of clipped, shows the most severe damage. It is essentially like area C of both experiments, but the degree of injury and other induced changes are even more severe. At the end of 17 days the epidermis in area D of the second experiment is still hyperplastic and acanthosis and utricular cysts are commonly found.

Conclusions. The combined treatment of methylcholanthrene-in-benzene painting plus 1000 r irradiation results in changes in the epidermis which are more severe than those occurring after either treatment alone. Sebaceous glands are destroyed by methylcholanthrene and redifferentiate only when hair growth is active. Where hair development is arrested or delayed by the irradiation, there is a failure of new sebaceous glands to redifferentiate until hair development again occurs. Furthermore, the initial damage to the epidermis in terms of hyperplasias and hyperkeratoses is less in areas where hairs were in anagen IV at the time of the single or combined treatment. Maximal damage occurs in telogen areas. Not only, then, is active hair proliferation associated with restoration, as in the case of sebaceous glands, but it also protects the epidermis from damage and it is associated with the repair of epidermal damage.

The explanation of such phenomena is as yet incompletely known. Cells of the external sheath of the follicle are directly involved in redifferentiation of sebaceous glands and to a considerable extent in repair of the epidermis. This layer of external sheath cells forms the morphological continuity between the hair germ or bulb and the basal layer of the epidermis. The resistance of epidermis to damage, as well as the capacity for repair, which are associated with hair proliferation may well indicate a comparable functional continuity of the external sheath. In any case, the importance of considering and controlling conditions in the skin at the time of and after treatments is obvious.

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Method for the Study of Tissue Metabolism *in Vivo* Using Fluoroacetate.* (18382)

V. R. POTTER, H. BUSCH,‖ AND J. BOTHWELL.
(With the technical assistance of H. Simonson.)
From the McArdle Memorial Laboratory for Cancer Research, University of Wisconsin, Madison, Wis.

Sodium monofluoroacetate was shown by Buffa and Peters(1,2) to produce marked accumulations of citrate in various organs of rats following injections of lethal doses of the compound. Potter and Busch(3) confirmed their findings for normal rat tissues and, in addition, showed that in several kinds of malignant tissue from rats, no citrate accumulated following the injection of fluoroacetate.

When fluoroacetate is administered, an enzyme necessary for citrate oxidation becomes inactivated, while the enzymes necessary for citrate production appear to be relatively unaffected(4-6). The continued production of citrate without adequate means for its further metabolism leads to the accumulation of citrate in some tissues to the extent of 30 to 50 fold over the normal operating level. Although the level of citrate that develops in any given tissue is the resultant of at least 5 separate factors(3) that have yet to be individually evaluated, it appears that *the rise in citrate is primarily an expression of the metabolism of the individual organ.* This conclusion is supported by the fact that the individual organs in the fluoroacetate-treated animal exhibit citrate values that differ widely from each other, and from the blood level (*cf.* Buffa and Peters(2), p. 494). Moreover, as will be shown(7), in combination with other inhibitors, the various tissues produce or fail to produce citrate in a manner that suggests lack of effective equilibration via the blood.

The present study was undertaken to extend the area covered by the previous reports in terms of both the dosage and the rate at which citrate accumulates. The object of the experiments was to develop an experimental approach to the problem of studying the operation of the Krebs citric acid cycle and ancillary reactions in individual organs *in situ.* The accompanying report(7) shows results obtained with animals under altered physiological conditions.

Experimental. Young male rats weighing from 180-240 g were obtained from the Holtzman Rat Co. Freshly prepared solutions of sodium monofluoroacetate (Monsanto) were injected intraperitoneally and the rats were allowed access to food and water either for a fixed period of time or until death was imminent. Parallel tests were made in some cases with purified fluoroacetate† but no significant difference was detected. It is doubtful that the biological tests would reveal the difference between 90% and 100% purity unless a highly toxic impurity were present. The dosages and times were varied in each experiment in order that each point would involve the use of different batches of animals. The animals were killed by decapitation and the organs were quickly removed and analyzed for citrate as previously described(3). The dosage of fluoroacetate was varied from 0.3 to 50 mg per kg and the time was varied from 45 minutes to 48 hours.

Results. The data for brain, heart, thymus,

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* This work was supported in part by the Jonathan Bowman Fund for Cancer Research.

‖ Public Health Service Postdoctoral Research Fellow of the National Cancer Institute.

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† Obtained through the courtesy of Dr. K. P. DuBois, Department of Pharmacology, University of Chicago.

spleen, and kidney are shown in Fig. 1. Data for the 0.3 mg per kg dose are omitted. The citrate accumulation was not followed beyond 2 hours in the case of the 5 mg dose, or beyond 6 hours in the case of the 2.0 and 3.5 mg doses for the reason that death occurred in the majority of animals that were observed longer than these times. A few animals at the 3.5 mg dose die before 3 hours, and an appreciable number die between 3 and 6 hours, so that the 6 hour animals are not average specimens. The LD_{50} on the basis of 24 hour survival lies between 1.0 and 2.0 mg per kg. With the former dose almost no deaths occurred before 24 hours and with the 2.0 mg dose there were no survivals after 12 hours.

The citrate accumulation was greatest at the 3.5 mg dosage, in all organs studied. The animals that received 5.0 mg per kg accumulated citrate as fast or faster than the animals receiving 3.5 mg but death prevented them from attaining the higher levels shown at the lower dosage.‡ Not shown in Fig. 1 are the results of a series in which 50.0 mg per kg was given to six animals. These animals survived for 22-70 minutes.‡ The average citric acid content of their tissues (μg per gram) was as follows: blood 186, brain 204, thymus 555, heart 877, lung 880, liver 46, kidney 1540, testes 108, spleen 291, pancreas 939 and muscle 171. These values show some marked deviations from the values obtained with 5.0 mg per kg (*cf.* Fig. 1 and previous reports) (2,3). At the 5 mg dosage the blood citrate was but slightly elevated (78, normals 58 μg per ml). However, at the 50 mg dosage the blood citrate was elevated to 186 μg per ml.

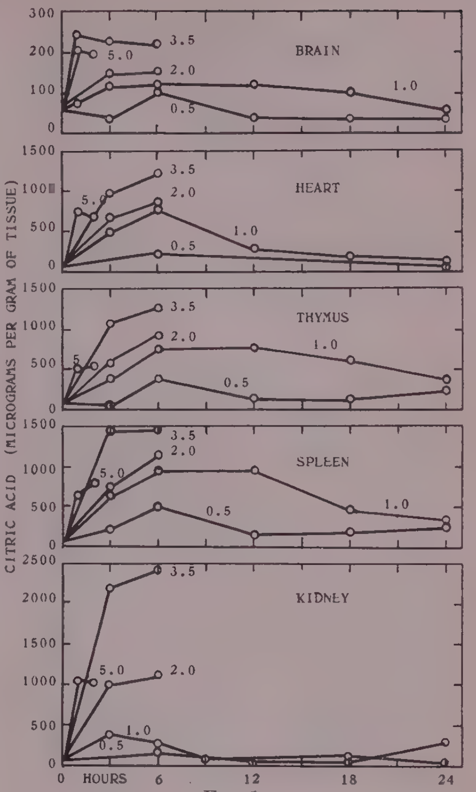

FIG. 1.

The citrate content of brain, heart, thymus, spleen, and kidney at various time intervals after the injection of doses of fluoroacetate ranging from 0.5 to 5.0 mg/kg. Each point is the average of 2 to 14 rats, usually 3 or 4 rats, with the major emphasis on the peak values. The curves for the various doses from 0.5 to 5.0 respectively represent the average values from 25, 32, 8, 18, and 11 rats respectively. The data on controls and on the 5 mg/kg dose are taken from a previous study(3).

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‡ Fig. 1 does not show any data on either liver or tumor tissue. Earlier studies(3) showed that neither of these tissues accumulated citrate following a lethal dose of 5 mg of fluoroacetate per kg. In order to supplement the earlier study, a number of experiments were carried out to determine whether a higher or lower dose of fluoroacetate would lead to citrate accumulation in these tissues. The 3.5 mg dosage was given to 2 rats bearing the Flexner-Jobling carcinoma and to 3 rats bearing the Jensen sarcoma, and the animals were killed at 6 hours. The average citrate values were 150 and 85 μg/g respectively in comparison with the 121 and 85 μg/g found in uninjected controls(3). When a still lower dosage was given, 0.5 mg/kg, to 3 rats bearing Flexner-Jobling tumors the citrate content at 6 hours was 118 μg/g. The high dosage of 50 mg per kg was given to 6 tumor-bearing animals and the normal tissues as well as the tumors were analyzed when death was imminent, as in the case of 6 non-tumorous rats described in the text. The results were about the same or slightly lower than in the non-tumorous animals (see text) except in the case of kidney in which the value was 644 μg/g in comparison with a value of 1540 μg/g in the non-tumorous animals, accentuating the trend seen earlier in tumor-bearing animals at the lower dose(3). The tumors contained less citrate than the tumors from the non-injected animals.

This finding suggests that at this dosage, possibly some organs were damaged sufficiently to become more permeable to citrate. This explanation may find support in the fact that the citrate content of the spleen was much lower than it was in the case of the lower dosage. The lung and pancreas, however, accumulated more citrate at the higher dosage.

Of considerable interest is a comparison of the relation between the dose of fluoroacetate injected and the amount of citrate accumulated in the different organs. If the data in Fig. 1 are visualized in a graph (not shown) in which the citrate per gram of tissue at 3 or 6 hours is plotted against the dose in mg per kg, it can be seen that for each tissue the amount of citrate accumulated rises with increasing dose and reaches its maximum level at a fluoroacetate dose of about 3.5 mg per kg. At higher doses, the plateau level is not attained, owing to the early death of the animals at doses of 5 or 50 mg per kg.

It is assumed that the maximum level represented by the 3.5 mg dose is a rough measure of the maximum capacity of the respective tissues to produce citrate, and since the level is about the same at 3 and 6 hours (Fig. 1) the best comparison of the ability of the various tissues to produce citrate may be obtained by analyzing the tissues 3 hours after the administration of 3.5 mg per kg. Further support for the use of this time period is obtained from studies(7) showing that the rate is almost linear on the basis of time periods of 1, 2, and 3 hours, and this combination of time and dosage constitutes the method herein proposed for further studies with supplementary studies on animals killed at 1 and 2 hours. Since the exact values are not apparent from Fig. 1, they are tabulated in Table I, which includes data from a concomitant investigation(7).

If the relation between citrate accumulation and dose of fluoroacetate is examined further, it may be seen that the shape of the resulting curves is not uniform from tissue to tissue. Kidney, which on the basis of results obtained at 3.5 mg per kg has the greatest citrate-forming potential, accumulates much less citrate at the 0.5 and 1.0 mg dosages than do the heart, spleen, and thymus. This result would not be unexpected if the latter tissues contained less of the citrate-oxidizing enzyme component that is inhibited by fluoroacetate and if the lower doses of fluoroacetate "titrated" a fixed amount of this enzyme from each tissue (*cf.* Ackermann and Potter)(8), since such a titration would leave a larger amount of effective citrate-oxidizing enzyme in the kidney tissue to prevent citrate accumulation at the lower levels of fluoroacetate. The fact that at the high dosages kidney accumulates more citrate than the other tissues suggests that it may actually have more of the enzymes involved in citrate formation and removal than do the other tissues.

TABLE I. Citrate Content of Various Rat Organs Following a Dose of 3.5 mg of Fluoroacetate per kg.

	Time after inj. of fluoroacetate			
Organ	0	1	2	3
Kidney	56(5)	557(3)	1440(4)	2211(7)
Heart	49(5)	636(3)	749(4)	988(7)
Thymus	55(5)	338(2)	620(3)	1070(6)
Spleen	59(5)	717(3)	1012(4)	1462(7)
Brain	57(5)	238(3)	189(4)	230(7)

Figures in parentheses indicate number of animals, data given in μg of citric acid per g of tissue, wet weight.

This marked difference between the response of kidney and other tissues may actually form the basis of a crude assay for fluoroacetate[§] since for example, the ratio of kidney

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8. Ackermann, W. W., and Potter, V. R., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 1.

[§] The question has been raised as to whether the liver and tumor tissue had been penetrated by the fluoroacetate, since they failed to accumulate citrate. Potter and Busch(6) showed evidence that the liver might shift the balance of citrate production: acetoacetate production in favor of the latter, thereby shunting fluoroacetate into acetoacetate rather than into citrate with a consequent return of the fluorine derivative to the blood-stream. By direct analysis, Hagan *et al.*(10) have shown that the liver contains somewhat less fluoroacetate than certain other tissues, following injections of fluoroacetate but no data were available on tumor nor was the method available. A bioassay was therefore attempted (*cf.* Frick and Boebel)(11) using the carcasses of the 6 citrate to spleen citrate is roughly 0.4, 1.0 and 1.67 at the 1, 2, and 3.5 mg doses of fluoroacetate, respectively.

The data in Fig. 1 show that the citrate content of the tissues falls with time, after the maximum has been reached, in the case of the non-lethal doses. This suggests that in the case of the higher doses, although no decline is seen, owing to the death of the animals, the apparent rise is merely the resultant of the rate of production and the rate of removal, so that the amount actually accumulated is not a full measure of the citrate-producing potential of the tissue. In independent studies elsewhere (personal communication from Dr. J. Schubert(9), Argonne National Laboratory) these 2 processes have been examined in greater detail, but the results of these studies have not been applied here.

Summary. Sodium monofluoroacetate was administered to Holtzman white rats in doses ranging from 0.3 to 50 mg per kg, and the citrate content of kidney, thymus, brain, spleen, heart, liver and tumors was determined at times ranging from 20 minutes to 48 hours. As in previous studies liver and tumors did not accumulate citrate, but the remaining tissues did, with the maximum occurring in all cases with a dose of 3.5 mg per kg at 3 to 6 hours after the injection. As a standard procedure this dose was recommended for the study of certain phases of the metabolism of individual tissues *in vivo*.

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rats that received the 50 mg/kg dose as a source of tissues. Twelve-gram portions of pooled tumor tissue, pooled livers, and combined hearts, spleens, lungs, and kidneys were fed, respectively, to 3 groups of 2 rats each, in the absence of other food. Eighteen hours later, the tissues had been completely eaten, and one of the tumor-fed animals was dead, having shown the typical signs of fluoroacetate poisoning. The remaining rats were killed, and the spleens and kidneys from the six rats (one postmortem), *cf* Buffa and Peters(2) were analyzed for citrate. The citrate content in μg per gram in the spleens was as follows: tumor-fed, 1404 and 790 (dead specimen); liver-fed, 1440 and 1130, pooled organ fed, 970 and 1310. The citrate contents in the respective kidneys were 457 and 242, 192 and 200, and 238 and 614. On the basis of the fact that the spleens contained more than the kidneys, in every case, it would appear that the fluoroacetate given was equivalent to an injected dose of less than 2.0 and more than 1.0 mg per kg, and that the tumors contained approximately as much as the other tissues.

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Sequential Blocking of Metabolic Pathways *in vivo*.* (18383)

Van R. Potter. (With the technical assistance of H. Simonson.)

From the McArdle Memorial Laboratory for Cancer Research, University of Wisconsin, Madison, Wis.

Malonate is well known as an inhibitor of succinic dehydrogenase in *in vitro* systems. Relatively few studies have been made of its action *in vivo*, and these have been limited to the metabolism of the organism in terms of blood(1,2) or urine(3,4) changes or to the phosphate balance in a single organ(5). Since malonate presumably should interrupt the citric acid cycle by blocking succinic de-

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* This work was supported in part by the Jonathan Bowman Fund for Cancer Research.

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hydrogenase, it should be possible to study the effect of malonate *in vivo* by measuring its effect upon the citric acid accumulation in individual organs in an animal treated with fluoroacetate, which has recently been shown to block the oxidation of citric acid *in vivo* (6-8).

The present study was undertaken as a model for what might result from the combined action, *in vivo,* of two inhibitors, each of which acts on the same metabolic sequence but upon *different* enzymes within a limited portion of the sequence. The combined use of two or more inhibitors in this manner will be referred to as *sequential blocking*.

In attempting chemotherapy by preventing the formation of an "essential metabolite" two inhibitors may be better than one from the following considerations. Both in the phenomenon of competitive inhibition by reversible inhibitors and in substrate "interference" in the case of irreversible inhibitors (9) an enzyme is less liable to combination with the inhibitor if its specific substrate is present in excess. When one inhibitor is used, the inactivation of part of the enzyme results in an accumulation of its substrate, which tends to protect the remaining enzyme from attack. However, the reaction might be slowed sufficiently so that if the next enzyme in the sequence is attacked by a second inhibitor the formation of its substrate by the prior enzyme may be so limited that the protection afforded to the second enzyme by its substrate becomes negligible. In this case the inactivation of the second enzyme should proceed farther toward completion, and the formation of product from the sequentially blocked system should be less than could have been accomplished with either inhibitor alone.

The study of sequential blocking *in vivo* requires a fairly complete knowledge of the individual steps and converging pathways in a metabolic sequence, the availability of effective inhibitors for at least two of the steps, and satisfactory analytical methods for the accumulating metabolites or the inhibited enzymes. There are indeed relatively few instances in which these requirements are fulfilled. In the case of the Krebs cycle, the ease with which citrate may be determined, the availability of malonate and fluoroacetate as inhibitors, and the immense amount of literature on the main metabolic pathway and its side reactions made the experimental manipulations in this study fairly simple. Nevertheless, it was found that the results obtained could not have been fully predicted on the basis of existing *in vitro* data.

The nature of the Krebs cycle is such that the product of citrate oxidation is ultimately converted to oxalacetate, which combines with acetate(C_2) to form more citrate, and, in the absence of ancillary reactions that produce the acids of the Krebs cycle, the amount of citrate that could accumulate would be no larger than the summation of these intermediates(10). The amounts of citrate that are actually found(6-8) are so large in some tissues that it might be assumed that proteins or other sources were, in fact, being mobilized as precursors of the Krebs intermediates during the development of the fluoroacetate block. On the basis of the known side reactions, these precursors might enter the Krebs cycle either between citrate and succinate or between fumarate and oxalacetate.

If sodium malonate acts *in vivo* according to what might be expected from *in vitro* studies, its primary effect should be to interrupt the Krebs cycle at the point of the conversion of succinate to fumarate. Thus the proper dosage of sodium malonate might be expected to alter citrate production in the presence of a fluoroacetate block in a manner that would depend upon the nature and extent of the side reactions. The present study shows that malonate affects citrate production differently in various organs and thus leads to the inference that the contribution of the side reactions differs widely from one

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organ to another.

Experimental. Young male rats weighing from 180 to 240 g were obtained from the Holtzman Rat Co. Neutral 1 M solutions of sodium malonate were injected subcutaneously in several sites on the back, following the indications from previous studies(1-5). The concentration was not varied but the dose was varied and increased beyond what had been used hitherto. Preliminary trials with *potassium* malonate intraperitoneally resulted in almost immediate deaths, and no experiments of this type are reported. Freshly prepared solutions of sodium monofluoroacetate (Monsanto) were injected intraperitoneally at a dosage of 3.5 mg per kg in all cases, either into rats that received no other treatment, or into rats that had received sodium malonate. This level of fluoroacetate was shown to result in the maximum rate of citrate production during the first 3 hours(6). In general, one animal was injected with fluoroacetate alone and tested in parallel with 2 or 3 animals that had received additional treatment. The animals were decapitated at 1 to 3 hours after the fluoroacetate injection, and the organs were quickly removed and chilled as before(6). In this study, only the heart, brain, kidney, thymus and spleen were removed for analysis. Protein-free filtrates were analyzed as before, using the modified pentabromoacetone method(8,11). Results are expressed in terms of micrograms of citric acid per gram of fresh tissue.

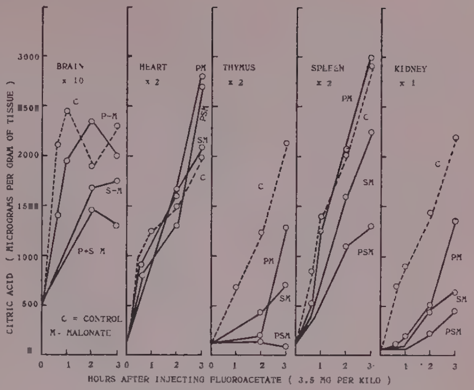

FIG. 1.

Citric acid accumulation in rat tissues following *sequential blocking* of the Krebs citric acid cycle by various combinations of malonate and fluoroacetate. Animals receiving fluoroacetate only are called controls, and the data are given in dashed lines labeled C. Malonate was given in various combinations in addition to fluoroacetate. Curves labeled PM (prior malonate) represent 1.2 cc of 1 M malonate per 100 g 60 min. prior to the fluoroacetate. Curves labeled SM (simultaneous malonate) represent the same dose of malonate, but the malonate was given at the same time as the fluoroacetate. Curves labeled PSM (prior and simultaneous malonate) represent a dosage of 1.2 cc of 1 M malonate per 100 g followed in 60 min. by *one-half* this amount of malonate and the usual dose of fluoroacetate. The data have been multiplied by the numbers indicated for each tissue in order to use the same scale for the ordinates. Each point represents the average of 2 to 14 animals (usually 3) and the control, PM, SM, and PSM curves represent 17, 5, 9 and 5 animals respectively, except in the case of thymus in which 12, 5, 4, and 5 analyses were obtained, owing to insufficient samples in the remaining animals.

Results. The results of the experiments with combinations of malonate and fluoroacetate are given in Fig. 1.[†] The first point to be emphasized is that the 5 different tissues responded individually just as in the case of fluoroacetate alone, again suggesting that the results depend essentially on the metabolism of the individual tissues. Thus in the malonate treated animals, the data have to be discussed for each tissue. The fact that strikingly different results were obtained suggests that no individual result can be ascribed to a general systemic effect.

The most decisive malonate block of citrate formation was obtained in the case of *thymus* when 1.2 ml of 1 M malonate was followed

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[†] Fig. 1 represents only the data obtained with the more effective doses of malonate and fluoroacetate. A number of tests were carried out with lower doses of both inhibitors. Two animals received 0.8 cc of 1.0 M malonate per 100 g followed immediately by the standard 3.5 mg dose of fluoroacetate. Three hours later the average citric acid values were: brain 215, kidney 1000, thymus 720, spleen 1315, and heart 1200. The kidney and thymus values are significantly lower than the controls and all of the values support the findings in Fig. 1. Tests with lower doses of malonate in combination with 1 mg of fluoroacetate were inconclusive. When 1.2 cc of malonate per 100 g was injected and no fluoroacetate was given, the citrate values were not elevated (27-49 μg per gram).

in one hour by one-half this dose with the fluoroacetate given at the time of the second malonate injection (curves labeled PSM, prior and simultaneous malonate, in Fig. 1).

When only one dose of malonate was given, the results depended on the time of administration: the *prior* dose of malonate (PM) suppressed citrate formation for two hours, but then wore off, indicating that the effective malonate concentration was maintained for a total of only 3 hours following a single subcutaneous injection. This conclusion is in harmony with the recent report that malonate can be metabolized in mice but that the CO_2 arising therefrom appears in the first 2 to 3 hours(12). Similar experiments on rats, including tests on urine would be of interest. When the *simultaneous* dose of malonate (SM) was given there was evidently some citrate synthesis before the malonate block became effective. Only in the case of the PSM dosage was the block fully effective.

Of the other tissues studied, only the *kidney* yielded data resembling that obtained from thymus. In the case of kidney the same phenomena were observed but the block was quantitatively less complete. In the case of *spleen* the prior malonate (PM) dose was without effect although the PSM dose showed a significant decrease in citrate accumulation. *Brain* showed a response that was very much like that of spleen except that the total amounts of citrate were much smaller at all combinations.

Heart showed a striking deviation from the other tissues. When prior malonate was given, the citrate formed during the third hour after fluoroacetate was markedly *increased* above that found in the animals receiving fluoroacetate alone. This finding suggests that in heart, oxalacetate may be formed from a source other than succinate under these conditions.‡ Had the PM or PSM dosages resulted in citrate accumulations no greater than in the controls it might have been possible to suggest that the amount of succinate formed in the malonate system was great enough to break through the competitive inhibition which is characteristic of malonate *in vitro*. However, the finding of a significantly *greater* citrate than in the case of fluoroacetate alone is not satisfactorily explained in these terms.‡ A clue to the alternative source of oxalacetate stems from experiments on fasting animals. In fasting rats, the injection of fluoroacetate results in a greater citrate accumulation in heart than is observed when fed animals are injected(13). The heart utilizes fatty acids(14), and moreover hearts from fasting(15) and diabetic(16,17) animals have higher than normal glycogen levels, while injected ketone bodies lead to increased heart glycogen values(18). Since malonate has been reported to induce ketonemia(1), the question arises as to whether, *in the heart,* ketone bodies may be convertible to both glycogen and to citrate by a pathway involving oxalacetate but not succinate.

The heart findings are of interest from another standpoint, since regardless of the explanation of how the oxalacetate became

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12. Lifson, N., and Stolen, J. A., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 451.

‡ Among the well-known sources of oxalacetate that are independent of succinate oxidation are (1) the transamination between aspartic acid and α-ketoglutaric acid to yield oxalacetic and glutamic acids, and (2) the Wood-Werkman reaction, or its modifications, by which pyruvate and CO_2 lead through a condensation reaction to oxalacetate. Since it was difficult to see how these reactions would be accelerated in the presence of malonate our attention was led to a consideration of ketone body formation, a reaction that is *increased* in the presence of malonate in liver *in vitro* (*cf.* Recknagel and Potter, to be published) and on this basis ketone bodies were discussed in the above text. Isotope experiments are in progress which may help to decide which, if any, of the 4 possible sources of oxalacetate predominates in the hearts of the malonate-poisoned animals.

13. Potter, V. R., and Simonson, H., unpublished.

14. Cruickshank, E. W. H., and Kosterlitz, H. W., *J. Physiol.*, 1941, v99, 208.

15. Lackey, R. W., Bunde, C. A., and Harris, L. C., *Am. J. Physiol.*, 1946, v145, 470.

16. Lackey, R. W., Bunde, C. A., Gill, A. J., and Harris, L. C., PROC. SOC. EXP. BIOL. AND MED., 1944, v57, 191.

17. Siliprandi, N., and Siliprandi, D., *Boll. Soc. Ital. Biol. Sper.*, 1946, v22, 1167.

18. Lackey, R. W., Bunde, C. A., and Harris, L. C., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 433.

available for citrate synthesis in the heart, it apparently was not available to kidney or thymus via the bloodstream.

Discussion. The results of these experiments are complicated by the fact that fluoroacetate *per se* does not inhibit citrate oxidation but has to be converted to some compound (possibly fluorocitrate or a derivative of it)(8,19,20) that is the true inhibitor. However, in the malonate experiments for example, where the data indicate a failure to form citrate, a failure to form fluorocitrate would yield the same result, the difference being that in the one case the citrate oxidation would be impaired while in the other it would not. This point can be tested experimentally by studying the oxidation *in vitro* using tissues from the poisoned animals. Busch and Potter(10) found a marked decrease in the ability of kidney homogenates from fluoroacetate-treated animals to oxidize citrate, without a concomitant decrease in the rate of oxidation with pyruvate plus fumarate. Further tests, including thymus, remain to be made.

In these experiments it has been tentatively assumed that malonate blocked succinate oxidation in all tissues, but the results in certain tissues might lead to alternative hypotheses that in these tissues malonate did not enter the cells, or was rapidly destroyed, or was overwhelmed by accumulating succinate. While the determination of succinate and of malonate is not as feasible as that of citrate, it is hoped to determine the amounts of these substances chromatographically. Earlier *in vivo* studies do not provide an answer to the tissue differences, but do indicate that succinic dehydrogenase was blocked, because increased amounts of succinate appeared in the urine(3,4).

It should be emphasized that the failure to form citrate after some pretreatment that is followed by fluoroacetate does not imply that the pretreatment, *e.g.* malonate, necessarily inhibited some enzyme in the metabolic sequence immediately prior to citrate. It was understood at the outset that the experimental basis for sequential blocking would have to be fairly well established by independent studies before a sequential block could be assumed to have occurred. The fluoroacetate block might result in no citrate formation in a tissue in which the cells have received lethal or near lethal treatment of any kind, even though the animal as a whole is destined to die much later. Through the cooperation of Dr. K. P. DuBois(21), unpublished experiments may be cited in which rats that received lethal doses of 800 r of X-ray irradiation, were given the standard fluoroacetate block (3.5 mg per kg and killed 3 hours later) at various times after irradiation. Non-injected animals died 4 to 5 days after irradiation. The injected animals were found to have a marked decrease in citrate production in spleen and thymus, a definite decrease in the case of kidney, and no effect in heart or brain. These changes began within 0 to 3 hours after irradiation and continued until death. It seems likely that in this and numerous other instances a destruction of some cell component will have repercussions that may involve the Krebs cycle and be revealed by the technic herein described before pathological changes will be evident microscopically. It would not be appropriate to consider such phenomena as sequential blocking in the absence of further information as to which specific enzyme, if any, is involved. However, the data illustrate another example of the usefulness of the fluoroacetate block in revealing differences in the metabolism of individual organs in animals following some treatment administered to the whole animal.§ Further studies to be reported later have been carried out in this laboratory using the fluoroacetate block in fasting animals and following the administration of aminopterin, alloxan, cortisone, and insulin. In all cases, the individual organs do not give parallel responses. It appears that the technic may have wide applicability in the study of tissue metabolism and in combination with iso-

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19. Elliott, W. B., and Kalnitsky, G., *J. Biol. Chem.*, 1950, v186, 487.

20. Liebecq, C., and Peters, R. A., *Biochem. and Biophysica Acta.*, 1949, v3, 215.

21. Du Bois, K. P., Cochran, K. W., and Daull, J., PROC. SOC. EXP. BIOL. AND MED., in press.

topically labeled precursors should permit the determination of the rate of entry of a given metabolite into the Krebs citric acid cycle in individual organs.

From the therapeutic standpoint, combinations of inhibitors or inhibitors and hormones that act sequentially on metabolic pathways such as protein and nucleic acid synthesis should be sought. Not only should simultaneous injections (*cf.* Skipper)(21) be attempted, but the individual drugs should also be given separately and in different sequences of administration.

Summary. The action of two or more inhibitors, each of which acts on the same metabolic sequence but upon different enzymes within a limited portion of the sequence, was defined as *sequential blocking*. As a model of such a system, the combined action of malonate and fluoroacetate was studied *in vivo*. It was shown that malonate decreased citrate formation to varying degrees in thymus, kidney, spleen and brain, but increased citrate formation in heart following the injection of fluoroacetate into rats.

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§ Note added Jan. 30. 1951: Livers from irradiated male rats were found to accumulate large amounts of citrate following the fluoroacetate treatment(21) in contrast to livers from normal male rats, which do not accumulate citrate. It was also found that normal *female* rats showed a liver response to fluoroacetate and the latter finding has been confirmed in this laboratory, in which preliminary tests with *males* given dessicated thyroid have given liver responses in 4 out of 9 animals, suggesting that several hormone relationships may be involved.

21. Skipper, H. E., *Cancer,* 1949, v2, 475.

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Effect of Yolk Removal in Day-old Chicks on Early Nutritional Requirements.* (18384)

H. MENGE, R. E. MORENG,† AND G. F. COMBS.
(Introduced by H. R. Bird.)

From the Department of Poultry Husbandry, University of Maryland, College Park.

Several investigators have found a positive relationship between early chick growth and the diet of the dam. Rubin and Bird(1) demonstrated that an unidentified growth factor present in cow manure, later shown to be identical with vit. B_{12}, was transmitted through the egg and thence to the chick. Subsequently, Rubin and Bird(2) reported the chick growth factor to be chiefly concentrated in the yolk of the egg. Bethke *et al.*(3), McGinnis and Carver(4), Wiese *et al.*(5), Robblee *et al.*(6), and Csonka and Olsen (7) also found that the diet of the breeders had a definite influence on the growth of the progeny. It is evident, therefore, that nutritional studies involving the chick are limited by the extent to which the chick can be depleted of the nutrients under consideration. Studies concerning vit. B_{12} and unidentified chick growth factors conducted by Menge *et al.*(8) in this laboratory have involved the use of chicks from dams fed rations containing no animal protein supplements and maintained on raised wire floors. Nevertheless, a preliminary period of from 1 to 2 weeks was usually necessary to further deplete the chicks and to standardize the effect of "carry-over" of the nutrient being investigated.

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† Present address: Poultry Department, North Dakota Agricultural College, Fargo, North Dakota.

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3. Bethke, R. M., Pensack, J. M., and Kennard, D. C., *Poultry Sci.,* 1947, v26, 128.

4. McGinnis, J., and Carver, J. S., *Poultry Sci.,* 1947, v26, 457.

5. Wiese, A. C., Petersen, C. F., and Lampman, C. E., *Poultry Sci.,* 1948, v27, 466.

6. Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *Poultry Sci.,* 1948, v27, 442.

7. Csonka, F. A., and Olsen, M. W., *J. Nutrition,* 1949, v39, 485.

Parker(9), and later Sloan(10) developed a technic for the removal of the yolk from day-old chicks. Sloan termed the operation "deutectomy" and postulated that this procedure could be very well adapted to nutritional studies. A simplified technic for the removal of the yolk from day-old chicks was developed, therefore, to render them more nearly deficient in the nutrients transmitted from the dam. The present paper describes this yolk-removal technic together with the subsequent effect on the early nutritional requirement for vit. B_{12}.

Method of yolk removal. The operative technic as employed for the removal of the yolk from the day-old chick is as follows: The chick is grasped by the legs and placed on its back with the umbilicus toward the operator. It is held in this position with its legs well forward during the entire operation. A solution of 0.5% phenol in 95% alcohol is used to saturate a small area about the umbilicus to sterilize the locus and keep the down in place during the operation. The solution is also used to clean the instruments at regular intervals. A small pair of tweezers is inserted into the umbilicus to enlarge the opening, and an incision of approximately one-eighth inch is made in the anterior portion with small, sharply-pointed scissors. The entire opening, including the umbilicus, should not be over one-fourth inch in length. If the incision is too long, difficulty will be experienced in keeping the intestines within the body cavity after the yolk sac has been removed. On the other hand, if the opening is too small, it may be quite difficult to tease the yolk sac through the incision without tearing. Extreme care must be exercised in making the incision so that only the skin, the muscular wall, and the peritoneum are cut, while the thin membrane of the yolk sac is left intact. The yolk membrane with its contents is usually visible after this incision is made, for it will tend to force its way through the opening after the peritoneum has been severed. The yolk is easily recognized by its yellow color. Every attempt is made to stimulate the chick to chirp, since this creates an internal pressure which forces the yolk through the opening.

The yolk sac is grasped by the fingers and carefully teased through the opening until the yolk stalk, which joins the yolk sac to the intestine, becomes visible. Instruments have been used with little success in the actual removal of the yolk because the yolk membrane itself is very delicate and is easily ruptured. A certain amount of tearing will be experienced, but in such cases the membrane can be removed with as much of its contents as possible and the remainder of the yolk material forced out of the body cavity by application of gentle pressure to the abdomen of the chick. A small spatula is placed over the cavity and at the juncture of the yolk stalk and intestines. The yolk stalk is then severed with a cauterizing needle. The spatula blade serves to prevent the intestine from being forced through the body cavity and also shields the intestine from the heat of the cauterizing instrument. The incision is then closed with a metal suture. Immediately following the operation, the chicks should be provided a good source of heat to lessen the effect of shock. Most of the chicks exhibit no signs of discomfort within an hour after deutectomy. The fact that the yolk sac is removed through a natural opening contributes to the success of the operation. There appears to be less chance of tearing the yolk membrane and the skin by utilizing the umbilicus in this fashion, for it is here that the muscles have been separated naturally to allow the yolk sac to enter the body cavity of the chick. This type of incision can also be easily closed. After a certain amount of practice, from 2 to 4 minutes were found to be required to deutectomize each chick. The mortality observed throughout was only 4%

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8. Menge, H., Combs, G. F., and Shorb, M. S., *Poultry Sci.*, 1949, v28, 775.

9. Parker, Sylvia L., *Hilgardia*, 1929, v4, 1.

10. Sloan, H. J., *Poultry Sci.*, 1936, v15, 23.

in excess of that noted in unoperated chicks.

Experimental. Two experiments were conducted with day-old New Hampshire chicks of mixed sexes to study the effect of deutectomy on their early requirements for vit. B_{12}. The chicks were reared in electrically heated batteries with raised wire floors. Feed and water were supplied *ad libitum.* The diet used had the following percentage composition: cerelose (glucose) 65.67, alpha protein 23, soybean oil 3, salts 1M[‡] 6, vit. A and D feeding oil (3000 A, 400 D) 0.5, glycine 1, DL-methionine 0.6, L-leucine 0.2, and protamone 0.03. Each 100 g of diet also contained the following amounts of vitamins (mg): thiamine HCl 1, riboflavin 1, Ca pantothenate 2, niacin 5, pyridoxine HCl 0.6, folic acid 0.15, para-amino benzoic acid 0.2, menadione 0.5, biotin 0.02, alpha-tocopherol 0.5, i-inositol 100, and choline chloride 200. Vit. A (1200 I.U.) and alpha-tocopherol acetate (0.5 mg) were also administered weekly by dropper. This diet, which is quite deficient in vit. B_{12}, was fed to all groups of chicks.

The chicks used in Exp. I were the progeny of hens receiving a ration containing 4% fish meal. Four uniform groups, each containing 20-day-old chicks, were employed. An experimental period of five weeks was used. The chicks in Groups 1 and 3 were deutectomized, whereas the chicks of Groups 2 and 4 were not. The chicks of Groups 1 and 2 received no supplementary vit. B_{12}. Each chick in Groups 3 and 4 received vit. B_{12} by intramuscular injection as follows: 0.6 γ on the first and eighth day and 1.2 γ on the 15th, 22nd and 29th day of the experiment. The chicks employed in Exp. II were from hens receiving a ration which contained no animal protein concentrates. Seventy-five chicks, 50 of which were deutectomized, were fed the vit. B_{12}-deficient diet for a 2-week experimental period. During this period each chick received 0.6 microgram of vit. B_{12} by intramuscular injection on the first and eighth day. At the end of this time, one group of unoperated chicks (Group 1) and two groups of deutectomized chicks (Groups 2 and 3) were selected. Each group consisted of 16 chicks, comparable on the basis of general appearance and body weight. During the 3-week experimental period, each chick in Groups 1 and 2 received intramuscular injections of vit. B_{12} as follows: 1.2 μg on the first and eighth day and 1.6 μg on the 15th day. Chicks in Group 3 received twice the amount of vit. B_{12} at each injection.

TABLE I. Effect of Deutectomy on Vit. B_{12} Requirement of Chicks.

Group No.	Type of chick	Total μg vit. B_{12} inj. per chick during exp. period	Avg gains[*] during experimental period
		Exp. I.	
1	Deutectomized	0	239 (17)[†]
2	Unoperated	0	316 (19)
3	Deutectomized	4.8	322 (18)
4	Unoperated	4.8	321 (18)
		Exp. II.	
1	Unoperated	4	261 (12)
2	Deutectomized	4	205 (13)
3	”	8	248 (14)

[*] Adjusted for sex differences.

[†] Numbers in parentheses refer to number of surviving chicks.

Results and discussion. The results of Exp. I and II are presented in Table I. In Exp. I the deutectomized chicks which received the vit. B_{12}-deficient diet alone (Group 1) grew less rapidly than did the unoperated chicks fed the same diet (Group 2). However, when vit. B_{12} was administered to deutectomized and unoperated chicks fed the same diet (Groups 3 and 4), no difference in average gain was obtained. It is evident, therefore, that removing the yolk from day-old chicks by means of deutectomy resulted in the removal of vit. B_{12} or another substance possessing vit. B_{12} activity. The results of Exp. II confirm the observations noted above since the deutectomized chicks required twice as much supplemental vit. B_{12} as did unoperated chicks in order to support a similar rate of growth. These observations are in agreement with Yacowitz *et al.*(11) and Milligan and Combs(12) who showed that vit. B_{12} activity is transmitted from the dam through the yolk of the egg. These workers also demonstrated that the diet of the dam had a direct influence on the amount of vit. B_{12} activity contained in the egg yolk.

[‡] Described by Briggs, G. M., *J. Nutrition,* 1946, v31, 79.

11. Yacowitz, H., Peeler, H. T., Miller, R. F., Carlson, C. W., Heuser, G. F., and Norris, L. C., Informal Poultry Nutrition Conference, Atlantic City, N. J., April, 1950.

The growth response of deutectomized chicks as compared with that of unoperated chicks in both experiments demonstrates that the yolk-removal operation had little or no effect on growth when the diet of the chick was adequately supplemented. Although no evidence was obtained in these trials to demonstrate the removal by deutectomy of nutrients other than vit. B_{12}, this procedure is considered to be of value as a means of reducing the initial "carry-over" of nutrients from the dam in day-old chicks.

Summary. An operative technic for the removal of the yolk from day-old chicks has been described. This procedure was developed to render chicks relatively free of the nutrients transmitted through the yolk from the dam. Observations on the growth rate of deutectomized chicks as compared with that of unoperated controls show that the operation had little or no effect on growth when an adequately supplemented diet was supplied. The results of these trials demonstrate that vit. B_{12} or a substance possessing vit. B_{12} activity was present in the yolk of the day-old chick and that it was removed by deutectomy.

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Critical Evidence that Vagal Stimulation Does Not Release Gastrin.

(18385)

HENRY D. JANOWITZ AND FRANKLIN HOLLANDER.

From the Gastroenterology Research Laboratory, The Mount Sinai Hospital, New York City.

At least 2 mechanisms have been shown unequivocally to result in the gastric secretion of hydrochloric acid: (a) A vagally mediated mechanism, which is physiologically activated by the thought, sight, smell and ingestion of food; by stimulation of the vagus nucleus chemically with insulin-induced hypoglycemia; or by electrical stimulation of the vagal trunks directly; and (b) a chemical mechanism, which is physiologically activated by the presence of food in the antrum of the stomach or by distention of the antrum and which is followed by the release of the hormone, gastrin. These mechanisms have generally been considered to be independent of each other(1) although, in the normal course of ingestion and digestion of food, they may operate synchronously as well as successively. In recent years, however, the view has been advanced that a more direct relation exists between the nervous and the hormonal phases of gastric acid secretion. Uvnäs(2) has presented evidence from which he concluded that vagal stimulation during the neural phase of gastric secretion leads to the release of the antral hormone, gastrin. This hypothesis is extremely attractive, since it unifies the

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1. Dragstedt, L. R., Woodward, E. R., Storer, E. H., Oberhelman, H. A., Jr., and Smith, C. A., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 676.

2. Uvnäs, B., *Acta Physiol. Scand.*, 1942, v4, Supplement 13.

various phases of gastric secretion by a common underlying neuro-humoral mechanism, and this possibility deserves careful consideration. Uvnäs employed anesthetized dogs and cats, and stimulated the cervical vagi electrically. His findings, as summarized by himself, fall into 2 main groups: First, the gastric secretion of HCl during vagal stimulation is greatly diminished if the pyloric region is deprived of its arterial blood supply, if the venous blood flow from that region is obstructed, if the function of the pyloric mucosa is depressed by cocainization, or if the pyloric portion of the stomach is resected. Second, cross circulation experiments were interpreted as indicating that a gastric secretory agent is liberated from the pyloric region during vagal stimulation. These findings have been restated by Kahlson, without essential addition of experimental evidence(3). Although Babkin and Schachter(4) failed to confirm the studies of Uvnäs, the suggestion that vagal stimuli release gastrin still continues to appear in the literature from time to time(5). More recently, Linde(6) has reported his repetition of Uvnäs' experiments using anesthetized cats, in which secretion of HCl induced by vagal electrical stimulation was diminished, but not completely abolished by (a) resection of the pyloric region, (b) ligation of the arteries supplying this part of the stomach, and (c) cocainization of the pyloric mucosa.

In the course of some experiments performed in this Laboratory(7), and designed to compare insulin and food as stimuli in the differentiation of vagal and non-vagal canine gastric pouches, evidence has been presented in part which bears directly on the question at hand. The pertinence of these studies to the problem of the vagal release of gastrin has been commented on by Grossman in his recent comprehensive review of gastrointestinal hormones(8) and by Linde(6). Because the significance of these experiments does not seem to have been generally recognized, and because of the questions raised by these latter reviewers, the present report was designed to furnish critical evidence that vagal stimulation does not release gastrin in the dog.

Methods. Four mongrel dogs with vagally denervated (Heidenhain) pouches, were employed in this study. One of the vagotomized pouches was prepared from a vagal pouch (Hollander-Jemerin type), one was converted from a Pavlov pouch, one was prepared by the conventional Heidenhain technic, the fourth was prepared as a Pavlov pouch but had suffered destruction of the vagal fibers supplying it, so that it actually was of the Heidenhain variety. Vagal stimulation was induced by the intravenous injection of 1.2-1.5 units of insulin in all 4 dogs, and by sham feeding in one dog following esophagostomy. All dogs were fasted for at least 20 hours before each experiment. The experiments were started only when the secretory activity of the pouch had reached basal anacidity, *i.e.*, 2 successive 15-minute samples were less than 0.2 ml in volume and contained no free HCl. Adequate hypoglycemia was considered to have been secured if the blood sugar was depressed to 50 mg/100 ml or below. Acidity was determined by colorimetric titrimetric procedures previously described from this Laboratory(9). Blood sugars were determined in duplicate by the method of Folin-Wu, as given by Reiner(10).

Results (Summarized in Table I). These 4 dogs were stimulated 11 times with an adequate insulin-induced hypoglycemia. In all instances, 0.1-0.3 ml of viscous mucus was obtained from the pouches during 2½ hours of collection, but at no time was even a trace of acid elicited.

Dog No. 118 was then subjected to esophagostomy, and vagal stimulation was induced

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3. Kahlson, G., *Brit. Med. J.*, 1948, v2, 1901.

4. Babkin, B. P., and Schachter, M., *McGill Med. J.*, 1944, v13, 127.

5. Glass, G. B. J., and Wolf, S, PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 535.

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7. Jemerin, E. E., Hollander, F., and Weinstein, V., *Gastroenterology,* 1943, v1, 500.

8. Grossman, M. I., *Physiol. Rev.*, 1950, v30, 33.

9. Hollander, F., *J. Biol. Chem.*, 1931, v91, 481.

10. Reiner, M., Manual of Clinical Chemistry, Interscience Publishers, Inc., New York, 1941.

TABLE I. Response of Vagally Denervated Canine Fundic Pouches to Vagal Stimulation of Main Stomach.

Dog No.	Exp. No.	Stimulus	Lowest blood sugar (mg/100 ml)	Pouch response			Comment
				Vol. (ml)	Character	Acidity	
46	74	Insulin	30	.2	Viscous mucus	0	Pouch responds to food and histamine by acid secretion
	78	,,	29	.2	,,	0	
74	45	,,	42	.3	,,	0	Same
	46	,,	43	.3	,,	0	
	47	,,	30	.2	,,	0	
108	63	,,	31	.3	,,	0	Same
	64	,,	43	.2	,,	0	
	68	,,	36	.1	,,	0	
118	75	,,	20	.3	,,	0	Same and simultaneously, main stomach responds to insulin hypoglycemia by acid secretion
	76	,,	28	.1	,,	0	
	79	,,	18	.1	,,	0	
118	80	Sham feeding		0			Same and simultaneously, main stomach responds to sham feeding by acid secretion
	81	,, ,,		0			
	82	,, ,,		0			
	83	,, ,,		0			

by sham feeding. In 4 experiments with 30 minutes of sham feeding with cooked horse meat, there was again no secretory response in the vagally denervated pouch.

The ability of all 4 of these vagally denervated pouches to secrete acid on the same day under other conditions was demonstrated by copious acid secretion following histamine given subcutaneously. The ability of the pouch to secrete in response to chemical stimulation of the main stomach was proven by large amounts of acid in the pouch following a meat meal. The adequacy of the vagal stimulation of the main stomach under the test conditions was confirmed in one of the dogs (No. 118) by the presence of large amounts of free HCl aspirated from the main stomach during both insulin hypoglycemia and sham feeding.

Discussion. These experiments indicate clearly that intense vagal stimulation of the main stomach by insulin-induced hypoglycemia, or by sham feeding in the esophagostomized dog, did not result in the secretion of any acid whatever, by the vagally denervated (Heidenhain) pouch. Hence, it must be inferred that intense vagal stimulation of the main stomach with its pylorus and antrum intact did not release any hormonal or humoral factors capable of stimulating the secretion of acid by the vagally denervated pouch. Grossman(8), in accepting these results as constituting "crucial evidence in disproof of the possibility that vagal stimuli release gastrin," added the proviso, "unless the Heidenhain pouches studied by Jemerin were too refractory to respond to gastrin." Linde(6) recognized also that this type of evidence "suggests that stimulation of the vagal secretory nerves does not lead to release of gastrin. However, the possibility exists that gastrin is really released, but in such small amounts that the pouch does not respond." In this connection, it may be pointed out that the pouches were shown to be capable of responding both to histamine, and to food placed in the main stomach. Further, if the secretion of the main stomach following vagal stimuli as suggested by Uvnäs is due to released gastrin, there does not seem to be any valid reason why the pouch should also not respond to the postulated circulating gastrin. Although the implications of our experiments seem clear cut, the conflicting evidence(2,6) previously cited, has not been completely explained. Perhaps such explanation is unnecessary, since Babkin and Schachter(4), in repeating the Scandinavian workers' experiments, failed to confirm them, and demonstrated acid secretion by vagal stimulation in the complete absence of the pylorus and antrum. These workers emphasized the importance of avoiding surgical trauma to the blood supply of the stomach.

This failure of vagal stimulation to release gastrin is consistent with the large body of experimental evidence which indicates that the mechanisms of the cephalic (neural) phase and of the gastric (hormonal) phase of acid secretion may operate quite independently of each other. This does not deny the possibility of additive or synergistic action between these two mechanisms. Nor does the present report controvert the evidence which indicated that local nervous pathways are involved in the release of gastrin by food in the stomach or by antral distention, but these are purely local(8).

Conclusion. The failure of vagally denervated canine gastric pouches to secrete acid in response to intense vagal stimulation of the main stomach constitutes critical evidence that vagal stimulation does not release gastrin.

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Metabolism of Sea Urchin Spermatozoa and Induced Anaerobic Motility in Solutions of Amino Acids. (18386)

ALBERT TYLER AND LORD ROTHSCHILD.
*From the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, Calif., and the Department of Zoology, University of Cambridge, England.**

The addition of any one of a number of different amino acids and peptides to suspensions of sea urchin spermatozoa in sea water has been found greatly to extend their functional life span(1). The period during which the spermatozoa retain their fertilizing capacity in dilute suspensions, can be increased more than 50-fold by means of these agents. During the period of prolonged life span the spermatozoa remain highly motile.

In contrast with mammalian spermatozoa, the metabolism of sea urchin spermatozoa is predominantly aerobic(2). In the absence of oxygen the spermatozoa soon become motionless(3,4). Ordinarily, the respiration of sea urchin spermatozoa decreases with time following dilution, from a high initial rate to practically zero, concomitant with loss of motility. It might, then, be expected that during the period of extended life and continuing motility induced by added amino acid or peptide the respiration would remain at a level similar to that of a freshly diluted suspension in ordinary sea water. The results of the present experiments show that such is not the case.

Material and methods. The sea urchins *Arbacia punctulata* (obtained at Woods Hole, Mass.) and *Lytechinus pictus* (obtained at Corona del Mar, Calif.) were used in this work. The semen was obtained by the KCl-injection method(5). Oxygen uptake was measured by means of Barcroft-Warburg manometers with conical, single-side arm vessels of 16 to 20 ml volume in which sperm suspensions of 3 ml final volume were generally used. Shaking was 85 to 95 c.p.m. and 3 to 4 cm stroke. The amino acid solutions were made isosmotic with sea water, at the pH of sea water, and diluted with sea water to the desired concentration. Since, in prolonged manometric experiments, growth of bacteria may be a factor in the results, determinations of their presence were made by microscopic examination and, in certain experiments, by platings on medium suitable for the growth of marine bacteria(6). In addition use was made of penicillin in many of the experiments, to check bacterial growth.

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* Part of these investigations were carried out at the Marine Biological Laboratory, Woods Hole, Mass., with the assistance of Mr. Richard Glass.

1. Tyler, A., *Biol. Bull.*, 1950, v99, 324.
2. Rothschild, Lord, *Biol. Rev.*, 1951, v26, 1.
3. Harvey, E. B., *Biol. Bull.*, 1930, v58, 288.
4. Barron, E. S. G., *Biol. Bull.*, 1932, v62, 46.
5. Tyler, A., *The Collecting Net*, 1949, v19, 19.
6. Waksman, S. A., Renszer, H. W., Carey, C. L., Hotchkiss, M., and Renn, C. E., *Biol. Bull.*, 1933, v64, 183.

Results. Use of penicillin. In dilute suspensions of spermatozoa the rate of oxygen uptake decreases with time to barely measureable values following which there occurs a rapid increase in rate. In concentrated suspensions the secondary increase may occur before the initial decline in rate has reached zero. While it has been suggested that the secondary rise might be due to autolysis, it seemed more likely that bacteria, which are visibly present in increasingly large numbers in aging sperm suspensions would be responsible for the phenomenon. In order to test this, as well as to provide a method for following the respiration of sperm suspensions for prolonged periods, the action of penicillin (Penicillin G. Potassium; Squibb) was examined. The tests showed that this agent, at a concentration of 50 units per ml, was effective in checking the growth of the marine bacteria and in preventing the secondary rise in respiratory rate of the aging sperm suspension.

Fig. 1 illustrates the results of one such experiment in which oxygen uptake was followed for 17 hours. While the rate of respiration of the sperm in ordinary sea water is seen to rise rapidly from 12 hours on, that in the penicillin-sea water remains at its low level. Bacterial counts, made from agar-plates of serial dilutions of the contents of the vessels at the end of the experiment (17 hours), showed an average of 1.7 x 10^8 per ml for the sperm suspension in sea water and 1.8 x 10^2 per ml for the suspension in penicillin-sea water. The latter value is of the same order of magnitude as the bacterial counts of the original sea water (5 x 10^2) and the penicillin-sea water (6 x 10^2). Similar low bacterial counts were obtained in eleven other sets of platings from 20-hour sperm suspensions in penicillin (50 units per ml) sea water. The penicillin does not, however, indefinitely suppress bacterial growth, and the concomitant rise in respiratory rate. This is presumably due to the appearance of penicillin-resistant mutants, or the growth of penicillin-resistant types originally present, or deterioration of the penicillin in solution. From the present results, it is clear that the addition of penicillin is of considerable utility in enabling prolonged measurements to be made on the respiration of aging sperm suspensions. It should, also, be noted that penicillin, at the concentration employed (50 units per ml), does not affect the respiration of the spermatozoa.

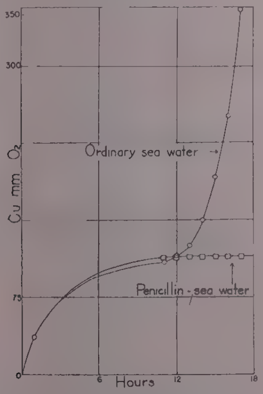

FIG. 1.

Oxygen uptake of senescing spermatozoa of *Lytechinus pictus* in ordinary sea water and in sea water containing 50 units of penicillin per ml. Suspension: approximately 2.5 × 10^8 spermatozoa in 3 ml. Temp., 22°C. See text for further data.

Action of amino acids. The respiration of aging sperm suspensions was examined in 25 sets of manometric experiments employing duplicate, triplicate, or quadruplicate vessels for the amino acid-treated and the control sperm. The amino acids tested were glycine, alanine and histidine. Tests of fertilizing capacity and examinations of motility were also made on the sperm suspensions from the respiration vessels. Since the results of the experiments were essentially similar, it will suffice to describe typical sets.

Fig. 2 illustrates the results of an experiment consisting of 3 control vessels of sperm in ordinary sea water and 3 vessels with the sperm in 0.05 molar glycine. The mixing of the sperm with the glycine solution, and with the corresponding volume of sea water, took place just after the first reading of the manometers by tipping the sperm from the side arm of the vessels. The readings began ½ hour after initial dilution of the sperm to 3 per cent suspension. The final concentration in the vessels was one per cent and the sperm count of the latter was 2 x 10^8 per ml. The

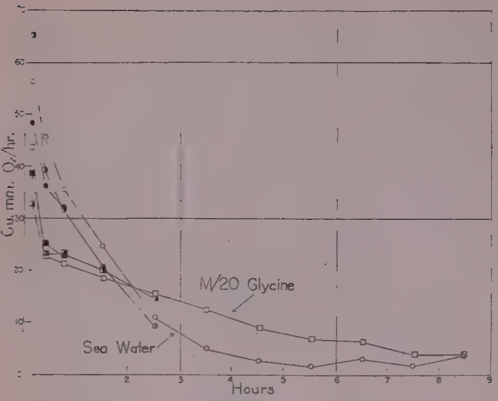

FIG. 2.

Rates of oxygen uptake of senescing suspensions of spermatozoa of *Lytechinus pictus* in sea water (circles) and in M/20 glycine in sea water (squares). Suspension: approximately 6 × 10^8 spermatozoa in 3 ml. Glycine or sea water (2 ml) mixed with sperm suspension (1 ml in side-arm) immediately after initial reading of manometers. Original dilution of semen at ½ hr before first reading. Temp., 22°C.

following features of the results may be noted. (a) Both glycine-treated and control sperm show a high initial rate of oxygen uptake which drops rapidly to relatively low values. (b) The initial rate of the sperm in glycine is markedly lower than that of the controls. (c) The rate declines less rapidly in glycine so that after the second hour the treated sperm respire at a somewhat higher rate than the controls.

In this experiment pairs of vessels were removed at 1, 3 and 9 hours at which time the sperm were tested for fertilizing capacity and examined for degree of motility. Fertilizing capacity was determined by inseminating aliquots of eggs with serially diluted amounts of sperm taken from the vessels and can be expressed, in terms of the reciprocals of the relative amounts of treated and control sperm required to give a definite percentage of fertilization (below 100%), as a percentage of the initial control value. The estimations of motility were subjective, but the figures given represent the judgment of three experienced observers and are believed to give a reasonably fair picture of the activity of the spermatozoa. The results of the tests and examination, given in Table I, show that the fertilizing capacity and motility of the control sperm drop off rapidly along with the decrease in respiratory rate. The sperm in glycine, however, remain highly active and retain high fertilizing capacity, as previously reported(1), although the respiratory rate also drops to a low level. Thus, after one hour the control sperm respire at a higher rate than do the sperm in glycine. The latter, however, exhibit considerably higher motility and fertilizing capacity. By 3 hours the control rate of oxygen consumption has dropped below that of the glycine-sperm. At this time the control sperm are almost all motionless and have very slight fertilizing capacity while that of the glycine-sperm remains high. At 9 hours the respiratory rate of the glycine-sperm has dropped to about 10% of its original value while fertilizing capacity and motility remain near their value at the start of the experiment.

These results are typical of the other experiments. A rather prolonged experiment is illustrated in Fig. 3. In this graph the cumulative values for oxygen uptake are given for a period of 34 hours for the sperm in glycine. The control sperm, in ordinary sea water, showed the secondary rise in rate of oxygen uptake starting at about 9 hours. The vessel was removed at 16 hours at which time tests of fertilizing capacity and motility showed the spermatozoa to be dead and mostly disintegrated with large numbers of bacteria present. In another control containing penicillin (50 units per ml) the secondary rise did not occur. Nevertheless, upon testing the spermatozoa at 16 hours they were found to be immotile and non-fertilizing. The glycine-

TABLE I. Results of Tests of Fertilizing Capacity and Examination of Motility for Respiration Experiment Illustrated in Fig. 2. (See text).

Time of testing, hr	Sperm suspended in	Relative fertilizing capacity, %	Degree of motility, %
1	Sea water	10 to 20	50
1	M/20 glycine	100	75 to 100
3	Sea water	<0.5	<0.1
3	M/20 glycine	100	75 to 100
9	Sea water	0	<0.01
9	M/20 glycine	90 to 95	50 to 75

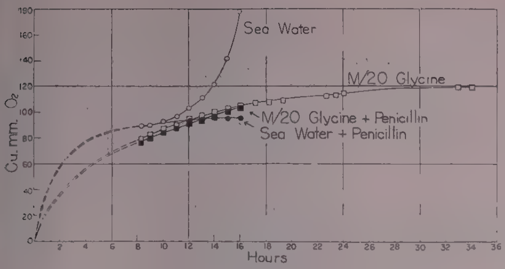

FIG. 3.

Oxygen uptake of senescing spermatozoa of *Lytechinus pictus* in sea water with and without glycine (M/20), and with and without penicillin (50 units per ml). Suspension: approximately 2 × 10^8 spermatozoa in 3 ml. Temp., 22°C.

sperm, with or without penicillin, showed no secondary rise in the respiratory rate, which from the 16th to the 34th hour, remained at the rather low average value of 0.4 cu mm per hour per 10^8 spermatozoa (*i.e.*, about 2.5% of the rate during the first hour). The motility of the latter at this time (34 hours) was about 50% and fertilizing capacity, compared with freshly collected sperm, of the order of 25%. It is of interest to note, too, that bacteria failed to grow, even without penicillin present, after 34 hours in the glycine-sperm suspension. This is understandable on the basis that no substrate is available for their growth until the sperm start to disintegrate. A similar explanation has been employed in studies(7) on the life span of unfertilized eggs.

It is clear from these experiments that the high initial rate of respiration exhibited by freshly diluted sperm is not a necessary concomitant of high motility and high fertilizing capacity. The spermatozoa in amino acid solution exhibit high motility and fertilizing capacity for a prolonged period during which their respiratory rate has dropped to a very low value. It might be supposed that the amino acid is metabolized in some nonoxidative manner or that it greatly enhances, somehow, the energetic efficiency of the normal oxidative processes. Previous experiments(1) showed no significant utilization of glycine by the sperm during prolonged incubation nor any significant production of ammonia therefrom. Further tests with carboxyl C^{14}-labelled glycine, kindly supplied by Dr. Henry Borsook, showed a negligible amount of decarboxylation.

In this experiment the weight and radioactivity of the carbon dioxide absorbed in the alkali well of a large respiration flask was determined for 20 ml of a 1% sperm suspension (5 x 10^9 spermatozoa per flask) in 0.05 molar glycine, containing the C^{14}-labelled glycine, for 4 and 8 hours of incubation. In the 8 hours a total of 639 cu mm of oxygen were consumed. From the C^{14}-content of the CO_2 absorbed in the alkali well approximately 0.0026 mg of the 75 mg of glycine present were decarboxylated; *i.e.*, about 0.0035%. A total of 3.8 x 10^{-5} moles of CO_2 (determined as $BaCO_3$) was absorbed in the alkali well of the respiration flask in the 8 hour period. Of this amount approximately 0.1% was derived from the glycine. The figures for the flask run for 4 hours were of the same order of magnitude.

It is noteworthy that the initial rate of oxygen consumption of the glycine-sperm is distinctly lower than that of the controls. This inhibition in rate is found to be even more marked when higher concentrations of the amino acid are employed. Fig. 4 illustrates an experiment in which glycine is tipped into the vessels from the side arm, at 30 minutes after the start, to give final concentrations of 0.16, 0.05, 0.01 and 0.002 molar. In the two higher concentrations the rate of

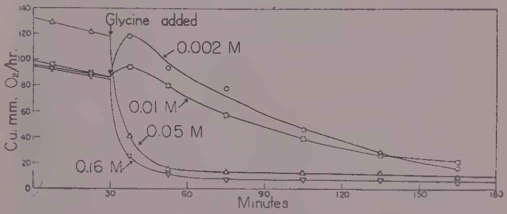

FIG. 4.

Action of glycine on rate of oxygen uptake of spermatozoa of *Lytechinus pictus*. At indicated time 1 ml of appropriate concentration of glycine in sea water added, from side-arm, to a suspension of approximately 9 × 10^8 spermatozoa in 2 ml of sea water, to give final concentrations listed. 50 units per ml penicillin present in all solutions. Temp., 22°C. See text for further data.

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7. Tyler, A., Ricci, N., and Horowitz, N. H., *J. Exp. Zool.*, 1938, v79, 129.

oxygen uptake drops considerably upon the addition of the glycine. In the two lower concentrations there is no initial depression in rate but rather a slight temporary rise (dilution effect). In this experiment, which ran for 18 hours (with 50 units per ml of penicillin present) the total oxygen uptake, relative fertilizing capacity, motility and number of bacteria at the end were as follows:

conc. of glycine	0.16 M	0.05 M	0.01 M	0.002 M
cu mm O_2 consumed	127	186	239	221
fertilizing capacity (%)	50- 75	50- 75	0	0
degree of motility (%)	75-100	75-100	0	0
bacteria per ml	420	240	760	820

The number of bacteria present is negligible as far as their contribution to the respiration is concerned. The total oxygen uptake at the end of the 18 hour period is much lower for the spermatozoa in the stronger than in the weaker glycine solutions. However, in the weaker glycine solutions respiration had almost ceased at this time while in the stronger solutions it averaged 4 to 5 cu mm per hour. In time, then, the higher totals for the latter would be obtained, as in other experiments employing concentrations of amino acid that give prolonged survival of the spermatozoa. The main points, to be noted in this experiment, are the depression of respiration and the continuing low rate in glycine solutions that maintain fertilizing capacity and motility.

Tests for anaerobic metabolism. In view of the low rate of oxygen uptake of the rather highly active sperm during the period of extended life span in amino acid solutions it seemed desirable to examine the possibility of the occurrence of appreciable glycolysis in the treated sperm. Under aerobic conditions sea urchin sperm produce relatively insignificant amounts of lactic, or other, acid and even less under anaerobic conditions with or without added sugar (8,9). In the present experiments determinations were made of anaerobic acid-production by glycine-treated sperm. The experiments were performed using ordinary sea water and an N_2 atmosphere in the vessels and also with bicarbonate enriched sea water in a 5% CO_2 in N_2 atmosphere. Penicillin (50 units per ml) was present in all the solutions. Only very slight CO_2-production was obtained. Thus, 10^9 spermatozoa in 4 ml of sea water gave rise to a total of 20 cu mm of CO_2 in 6 hours, and in glycine the total was less rather than more. The addition of glucose had no noticeable effect. In view of the absence of any indication of glycolysis the results of examination of the spermatozoa removed from the anaerobic vessels proved rather startling. While the spermatozoa in sea water were completely inactive and nonfertilizing those removed from the glycine flasks were highly active (50 to 75%) and capable of fertilization after 6 hours of anaerobiosis.

Anaerobic motility induced by glycine. While the activity observed after removal of the glycine-treated spermatozoa from the vessels might be attributable to a reactivation in the presence of oxygen it seemed desirable to examine the possibility of anaerobic motility. A gas tight chamber for microscopic examination was, therefore, constructed.[†] Two hanging drops of sperm suspension, one in 0.05 M glycine-sea water and the other in ordinary sea water were introduced into the chamber which was then filled with purified N_2. Determinations of the degree of anaerobiosis attained at various times after filling the chamber were not made. The residual O_2 is removed by the spermatozoa themselves. Under the conditions of the experiments the spermatozoa in the sea water drop usually became motionless in one-half hour after filling the chamber with nitrogen. A large fraction of this time was undoubtedly required for removal of oxygen from the chamber and the hanging drops. In striking contrast to the short duration of motility of the spermatozoa in the sea water drop, those in the glycine-containing drop remained highly active for periods of 4 to 6 hours.

Although it is difficult to put the following observations on a quantitative basis, we have

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8. Spikes, J., *Am. Naturalist*, 1949, v83, 285.
9. Rothschild, Lord, *J. Exp. Biol.*, 1948, v25, 353.

[†] We are indebted to Mr. James H. Berrian for the construction of this chamber.

on several occasions noted: first, that as anoxia develops, the spermatozoa in ordinary sea water swim extremely actively for a short period before becoming motionless; and secondly, that the spermatozoa in sea water containing glycine swim *more* actively under anaerobic than aerobic conditions.

It must be concluded that the addition of glycine to the sea water converts an aerobic cell into one capable of sustained mechanical activity under anaerobic conditions. The results reported in the previous section make it unlikely that there is sufficient glycolytic activity to account for the observed motility. In addition determinations of possible ammonia production and urea formation under anaerobic conditions yielded no significant quantities of these.

Discussion. The present results show that sea urchin spermatozoa suspended in sea water containing an amino acid remain highly motile while consuming oxygen at a very low rate compared with the initial rate of control sperm in ordinary sea water. The rate of oxygen uptake of a sperm suspension does not appear to be commensurate with the motility of the spermatozoa in the suspension. This implies that only a fraction of the oxygen uptake of a freshly diluted suspension is concerned with the liberation of the energy necessary for movement. This possibility is supported by the observation(9) that a considerable reduction in respiration can be effected with CO/O_2 mixtures in the dark without any corresponding reduction in motility. However, it is also clear, from the unexpected finding of anaerobic motility in the presence of glycine that sea urchin spermatozoa are not exclusively dependent upon a supply of oxygen for prolonged activity. As there is, even in the presence of glycine, an insignificant amount of anaerobic glycolysis or CO_2 production, negligible ammonia production, and no indication of urea formation, it seems improbable that glycine is utilized anaerobically as a substrate. Previous evidence(1) along with the present labelled glycine experiments make it very unlikely that it is so utilized under aerobic conditions. This view is supported, too, by the fact that, though the action of glycine is proportional to its concentration, those concentrations at which its effect is not optimal (*e.g.* 0.01 molar) would probably be high enough to achieve optimal enzyme saturation if the amino acid were acting as a substrate.

Since the amino acid does not appear to act as a substrate, it follows that its presence enables the spermatozoa to utilize their endogenous substrate for motility and maintenance of fertilizing capacity with more than normal efficiency. After motility has ceased in a suspension of sea urchin spermatozoa, the sperm still contain oxidizable material (9). Together with the present results, this means that spermatozoa do not normally die of substrate exhaustion.

Further experiments are needed to elucidate the mode of action of amino acids and peptides in achieving these results. One possible explanation centers around the role of trace metals, such as copper and zinc, in sea urchin sperm metabolism(10). The amino acid or peptide might exert their effect by forming complexes with trace metals in the medium. Experiments are in progress to investigate this and other possibilities.

Summary. 1. The metabolism of sea urchin spermatozoa has been examined in sea water containing amino acids which, as had been previously shown, greatly extend the functional life span of these cells. 2. The oxygen uptake of spermatozoa in sea water containing amino acid resembles that of spermatozoa in normal sea water in that a high initial rate is followed by a steady decrease. 3. In the presence of amino acid, however, the initial rate is lower than that of the controls, the degree of inhibition increasing as the concentration of the amino acid is increased. 4. The low rates of oxygen uptake attained after a period of senescence are significantly higher in the presence of amino acid than in the controls. 5. Suspensions of spermatozoa in sea water show a secondary rise in O_2 uptake shortly after a period of extremely low, or non-existent, oxygen uptake following loss of motility and fertilizing capacity. 6. This

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10. Rothschild, Lord, and Tuft, P. H., *J. Exp. Biol.*, 1950, v27, 59.

secondary rise is much delayed in the presence of amino acid, corresponding to the extended period of motility and fertilizing capacity. 7. The secondary rise is associated with the growth of bacteria as the spermatozoa die and disintegrate. 8. The addition of penicillin (50 units per ml) is efficacious in checking bacterial growth and the secondary rise in oxygen uptake in suspensions of senescing spermatozoa. 9. In the presence of amino acid, motility and fertilizing capacity can be maintained near their original level for many hours after the rate of oxygen uptake has dropped to less than 5% of the initial rate. 10. Previous evidence that the spermatozoa do not metabolically utilize the added amino acid to any appreciable extent is further supported by the results of experiments with carboxyl C^{14}-labelled glycine. 11. The total oxygen consumption of spermatozoa in the presence of amino acid exceeds that of the controls, which means that the death of the spermatozoa under ordinary conditions is not due to substrate exhaustion but rather to the inability to utilize fully their endogenous substrate. 12. While sea urchin spermatozoa in sea water are quickly immobilized by lack of oxygen, the presence of amino acids enables them to remain highly active for as long as 6 hours at room temperature. 13. No significant glycolysis, CO_2 production, production of ammonia or urea formation is found to occur anaerobically in the presence or absence of amino acid and glycolyzable sugar.

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Antibiotics in Mature Fowl Nutrition.* (18387)

J. V. HALICK AND J. R. COUCH.

From the Departments of Poultry Husbandry and Biochemistry and Nutrition, Texas Agricultural Experiment Station, College Station, Texas.

Olcese, Couch and Lyman(1) reported that the hatchability of eggs from hens fed a diet low in vit. B_{12} decreased to zero in 2 to 4 weeks and further that the hatchability of eggs from hens fed this diet, supplemented with APF concentrates, decreased about the ninth or tenth week of the experimental period. Subsequently Olcese and Couch(2) showed that the injection of crystalline B_{12} into eggs from hens fed a diet low in the vitamin promoted normal hatchability from the sixth through the eighth week but failed to do so after the ninth week in a 17-week experiment. The above-mentioned reports indicate that the hens were depleted of an unidentified factor which was required for normal embryonic development about the ninth or tenth week of the experimental period, since B_{12} was supplied either in the form of an APF concentrate or as the crystalline vitamin. Couch *et al.*(3) in a later report showed that liver fraction "L" contained the unidentified hatchability factor since the feeding of this substance maintained normal hatchability for a period of 16 weeks; whereas, hatchability of eggs from hens given weekly injections of B_{12} decreased after the twelfth week of the test. Cravens and Halpin(4) reported earlier that Liver "L" and fish solubles contained an unidentified factor required by the hen for egg production and hatchability. A number

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* Supported in part by Project RG 1862, Division of Research Grants and Fellowships, U. S. Public Health Service, National Institutes of Health, Bethesda, Md.

1. Olcese, O., Couch, J. R., and Lyman, C. M., *J. Nutrition,* 1950, v43, 71.

2. Olcese, O., and Couch, J. R., *Poultry Sci.,* 1950, v29, 612.

3. Couch, J. R, Olcese, O., Sanders, B. G., and Halick, J. V., *J. Nutrition,* 1950, v42, 473.

4. Cravens, W. W., and Halpin, J. G., *J. Nutrition,* 1949, v37, 127.

of workers have also reported that unidentified factors are required in addition to vit. B_{12} for chick growth(5-9). It should be stated further that antibiotics have recently been shown to have a stimulatory effect on chick growth(10,11).

With the latter reports in mind it appeared desirable to determine whether the antibiotics might have a favorable effect on the hatchability and vitamin B_{12} content of eggs from hens fed diets low in B_{12} and low in the unidentified hatchability factor.

Experimental. Single Comb White Leghorn hens that had been used in an earlier experiment were utilized for the present study. For 5 weeks preceding the start of this test these birds were fed a practical all-mash ration. Records of egg production and hatchability were kept during this period and only birds that were laying at a satisfactory rate and that were producing eggs that would hatch were used in the present experiment.

At the end of the pre-experimental period 48 hens were selected and were divided into 12 groups of 4 hens each. Individual egg production and hatchability records were used in making the group assignments. The basal diet (A-15) was composed of 63% sucrose, 5% dried whey, 22% soybean protein (alpha), 3% soybean oil, 2% fortified fish oil (3000A-400D) and 5% salts IV. The following amounts of vitamins were added in mg per kg: thiamine hydrochloride, 40; riboflavin, 6; calcium pantothenate, 15; niacin, 100; pyridoxine hydrochloride, 4; 2 methyl-1, 4 naphthoquinone, 0.5; alpha tocopherol, 3; folic acid, 2; biotin, 0.2; choline chloride, 2000; PABA, 20; and inositol, 1000. In addition 7.5 g methionine and 4 g glycine were added per kg of diet. The crude materials used in this study (liver extract, Lilly Laboratories; APF 4†, Lederle Laboratories; and Liver fraction "L", Wilson and Company) were assayed for B_{12} content by the method of Skeggs *et al.*(12). The liver extract contained 13 μg B_{12} per ml and the APF 4 and liver fraction "L" contained 0.6 and 1.24 μg B_{12} per g respectively.

In view of the fact that Couch *et al.*(3) had found that dried whey would improve egg production, 5% of this product was used in diet A-15 of the present study. The first group of 4 hens was fed diet A-15 unsupplemented. Groups 2 through 11, inclusive, were fed diet A-15 supplemented as shown in Table I. Supplements fed groups 10 and 11 were added to diet A-15 in place of an equivalent amount of sucrose. The hens in group 12 of this experiment were fed a practical all-mash diet. The practical diet contained: 42% ground yellow corn, 15% ground oats, 10% wheat bran, 10% wheat gray shorts, 2% meat and bone scraps, 5% dehydrated alfalfa leaf meal, 2% fish meal, 12% soybean oil meal, 1% bone meal, ½% salt, and ½% fortified fish oil (3000A-400D). Ten grams of manganese sulphate and 20 g BY-21 (riboflavin supplement, Commercial Solvents Corporation, Terre Haute, Ind.) were also added per 100 lb. All experimental diets were mixed fresh once each week, and were kept before the birds at all times. Oyster shell and tap water were supplied *ad libitum*. The management and experimental procedure with regard to the care and handling of the birds, insemination, incubation and mortality records were the same as that previously reported by Olcese, Couch and Lyman(1).

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† Stated to contain 2-4 mg aureomycin per g by the manufacturer.

12. Skeggs, H. R., Huff, J. W., Wright, L. D., and Bosshardt, D. K., *J. Biol. Chem.*, 1948, v176, 1459.

TABLE I. Effect of Vitamin B_{12}, Liver Extract, Antibiotics, Lederle APF, and Liver "L" on Hatchability and Vitamin B_{12} Content of Egg Yolks.

		Avg % hatchability		Avg vit. B_{12} content of egg yolks (mμg/g)	
Group	Supplement to basal diet (A-15)	2-4 wk	5-8 wk	2-4 wk	5-8 wk
1	None	25	0	1.4	0.9
2	2 μg B_{12} inj.*	38.4	14.2	7.5	5.2
3	0.5 ml liver extr. inj.*	50	33.3	7.2	4.1
4	Aureomycin HCl (66 mg/kg)	0	0	2.6	1.1
5	Aureomycin HCl (66 mg/kg) + 2 μg B_{12}*	26.6	10	5.8	5.7
6	Streptomycin dihydrochloride (66 mg/kg)	52.9	4.3	3.0	1.4
7	Streptomycin dihydrochloride (66 mg/kg + 2 μg B_{12}*	50	6.6	7.8	4.0
8	Procaine penicillin (G) (33 mg/kg)	5	0	2.3	1.3
9	Procaine penicillin (G) (33 mg/kg) + 2 μg B_{12}*	62.9	10	3.6	4.5
10	2% APF 4 (24 μg B_{12})	71.4	26.6	7.1	6.1
11	4% liver "L" (49.6 μg B_{12})	95.4	78.3	7.8	6.8
12	Practical	96.2	90.4	2.7	1.2

* Per hen per week.

Eggs were set aside on the first day of the hatching week for the determination of the vit. B_{12} content of the yolks. The egg yolks of individual hens within a group were pooled. Ten grams of the pooled sample were accurately weighed, 190 ml of NaAc buffer (pH 4.5) were added and the sample was blended in a Waring Blendor. An aliquot equivalent to 1 g of egg yolk was pipetted into a 125 ml Erlenmeyer flask and the flask was stoppered with a cotton plug. Vitamin B_{12} was liberated from the egg yolk by autoclaving for 30 minutes in the NaAc buffer at pH 4.5 at 15 lb pressure. After autoclaving, the pH of the sample was adjusted to 7.0 and the volume was made up to 100 ml. This solution was filtered and stored in a refrigerator at approximately 20° C. Dilutions for assay purposes were made from this stock solution. The B_{12} content of the egg yolks was determined by the method of Skeggs *et al.*(13).

Results and discussion. The average percentage hatchability of eggs from hens fed diet A-15 unsupplemented was 25 for the second, third, and fourth weeks (Table I). No live chicks were obtained from these hens after the fourth week of the experiment. This observation is in agreement with the work of Couch *et al.*(3) where diet A-15 was fed under similar conditions. It should be pointed out that the vit. B_{12} content of egg yolks from hens fed diet A-15 alone was lower than that of any other group in the test (Table I). The hatchability of eggs from hens injected with B_{12} (Group 2) was higher than that of those where no B_{12} was injected. There is little question but that the B_{12} content of egg yolks was increased by the injection of the vitamin into the birds (Table I). A further increase in hatchability was apparent when the hens were each injected with .5% liver extract (reticulogen) per hen per week (Group 3). The vit. B_{12} content of the egg yolks from these birds is approximately the same as that of those from Group 2 (Table I). The feeding of aureomycin and penicillin apparently assisted in the depletion of the birds of vit. B_{12} and possibly of the unidentified factor (Groups 4 and 8), since in the latter 2 cases the hatchability from the second through the fourth weeks, inclusive, is lower than that of birds not fed the antibiotic (Table I). When vit. B_{12} was injected and aureomycin was fed (Group 5) the hatchability was only slightly lower than that of Group 2. Hatchability of

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13. Skeggs, H. R., Nepple, H. M., Valentik, K. A., Huff, J. W., and Wright, L. D., *J. Biol. Chem.*, 1950, v184, 211.

eggs from birds fed penicillin and injected with B_{12} (Group 9) is higher than that of Group 2 for the second through fourth weeks, inclusive. The feeding of streptomycin (Groups 5 and 6) appeared to maintain a somewhat higher level of hatchability during the second through the fourth week, inclusive, than that of comparable groups (1 and 2, Table I). The feeding of 2% APF 4 (Group 10) produced a higher percentage hatchability than did the injection of vit. B_{12} (Group 2). Couch *et al.*(3) have reported earlier that this APF, which is a by-product from the manufacture of aureomycin, probably contains some of the Liver "L" hatchability factor, although these workers fed 5% of this APF to demonstrate this fact. The hatchability of eggs from birds fed 4% Liver "L" (Group 11) is approximately the same as that of those fed the practical all-mash diet during the second and fourth weeks, inclusive, and slightly lower during the fifth through eighth week, inclusive. From these data it is quite evident that Liver "L" contains a factor necessary for hatchability and embryonic development, which further substantiates the work of Couch *et al.*(3).

It should be pointed out that the injection of vit. B_{12} into the birds (Group 2, 5 and 7) produced egg yolks that contained approximately the same amount of vit. B_{12} (Table I). Attention is also directed to the fact that egg yolks from birds fed APF 4 (Group 10) and Liver "L" (Group 11) contained about the same quantity of vit. B_{12} as did egg yolks from hens injected with vit. B_{12} (Groups 2, 5 and 7). It is not possible to explain the decreased B_{12} content of egg yolks from birds fed penicillin and injected with B_{12} (Group 19); particularly during the second through the fourth week, inclusive.

The data on the B_{12} content of egg yolks from birds fed the practical diet is of considerable interest, since the B_{12} content of yolks from these birds is low when compared to that of egg yolks of birds injected with vitamin B_{12} (Groups 2, 5 and 7) or fed APF 4 (Group 10) or Liver "L" (Group 11). Yet the percentage hatchability of eggs from birds fed the practical diet was above 90% throughout the test. This practical all-mash diet has been used for the production of depleted chicks to use in B_{12} chick work. It is believed that the data obtained on the vit. B_{12} content of the egg yolks points to the fact that vit. B_{12} is not the limiting factor in this diet, although the egg yolks obtained were low in B_{12}. It does point to the fact that even though the egg yolks are low in B_{12} that the diet contains an adequate amount of the Liver "L" factor or provides a favorable media where such factor can be synthesized in the intestinal tract of the bird.

Summary. Data have been obtained on the effect of feeding aureomycin, penicillin, streptomycin and APF concentrate and Liver "L" in a diet low in vit. B_{12}. Hatchability of eggs from birds fed this diet unsupplemented decreases to zero after the fourth week. Injection of vit. B_{12} into the hens improved hatchability somewhat. The feeding of aureomycin and penicillin alone apparently assisted in the depletion of birds of vit. B_{12} and possibly of the unidentified factor. Liver "L" contains a factor necessary for normal hatchability and embryonic development. The B_{12} content of the egg yolks was determined by a standard microbiological assay. Injection of vit. B_{12}, feeding of an APF concentrate and the feeding of Liver "L" produced egg yolks that contained approximately the same amounts of vit. B_{12}. Hatchability of eggs from hens fed the practical all-mash diet was about 90% during the test period. Yet, the vit. B_{12} content of egg yolks from these birds was lower than that of similar birds injected with vit. B_{12} or fed a source of vit. B_{12} in the form of an APF concentrate or Liver "L."

Aureomycin hydrochloride, streptomycin dihydrochloride, penicillin, crystalline vitamins, and one APF concentrate (APF 4) used in this study were generously supplied by Merck and Company, Rahway, N. J. and Lederle Laboratories, Pearl River, N. Y.

Soybean protein (alpha) was obtained from Glidden and Co., Chicago, Ill.

Soybean oil was contributed by Dr. J. E. Hunter, Allied Mills, Inc., Libertyville, Ill., and Buckeye Cotton Oil Company, Cincinnati, O.

Liver fraction "L" was contributed by Dr. David

Klein, The Wilson Laboratories, Division of Wilson and Company, Chicago, Ill.

The methionine and glycine were obtained through the courtesy of Dr. Julius Johnson, The Dow Chemical Co., Midland, Mich.

The dried whey was supplied through the courtesy of the Western Condensing Co., Appleton, Wisc.

The liver extract (reticulogen) was supplied by Lilly Laboratories, Indianapolis, Ind.

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Effects of Oxygen, Analeptics, and Artificial Respiration on the Toxicity of Procaine.* (18388)

H. R. HULPIEU AND VERSA V. COLE.

From the Department of Biochemistry and Pharmacology, Indiana University, Indianapolis.

The introduction into the blood stream of procaine in sufficient amount to cause a sudden high concentration is thought to cause circulatory collapse or "cardiac death," while a gradual increase in blood procaine produces first a central stimulation which is followed by depression with eventual respiratory failure(1-4). If this be true the most frequent cause of death from slow intravenous infusions of procaine should be respiratory failure. Such has been reported to be the case (5-8), although cardiac and respiratory failure may occur almost simultaneously(2,9). Experiments designed to study the effect of anesthesia and the rate of injection on blood levels and toxic reactions during intravenous infusions of procaine permitted preliminary observations on the cause of death from such administrations. In 81 out of 83 experiments on dogs, the heart continued to beat for from 30 seconds to 8 minutes after respiratory movements ceased. In the other 2, heart apparently stopped at the same time as respiration. It is of interest that these 2 dogs were anesthetized with ether, which has been shown to sensitize animals to procaine(10,11). These observations stimulated us to study in more detail the cause of death from slow intravenous infusions of procaine and to evaluate experimentally the therapeutic effectiveness of analeptics, oxygen, and artificial respiration.

Experimental. Controls. Eleven experiments were run in which the dogs breathed air. Blood pressure and respiratory movements were recorded on a kymograph by means of a mercury manometer and a Manning pneumograph. The condition of the heart was also checked with a stethoscope and respiratory movements by close observation of the animal. Ether was administered to produce light anesthesia for from 30 to 45 minutes before the procaine infusion was started. The procaine hydrochloride was injected as a 1 to 5% solution in normal saline by way of the right femoral vein at a constant rate of 2 mg/kg/min. The infusion of procaine was continued until respiratory movements ceased, at which time it was stopped. The ether was discontinued when the procaine infusion was started and thereafter was administered in the minimal amount

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TABLE I. Oxygen and Analeptics on the Toxicity of Procaine.

		Mean			
			Survival time	Blood levels mg %	
Exp. procedure	No. of dogs	Time of resp. failure, min.	after resp. failure, min.	Procaine	P.A.B.A.
Ether-air	11	21.6 ± 4.3	1.7 ± .3	3.22 ± .3	0.66 ± .14
Ether-oxygen	10	44.3 ± 5.1	5.1 ± .5	5.78 ± .4	1.13 ± .10
Ether-oxygen nikethamide	11	37.8 ± 4.9	3.9 ± .35	4.65 ± .46	1.18 ± .10
Ether-oxygen metrazol	10	42.8 ± 5.8	3.6 ± .7	4.80 ± .4	1.29 ± .34
Ether-oxygen picrotoxin	6	38.0 ± 6.3	4.2 ± .5	4.93*	1.19*
Ether-oxygen lobeline	2	35.0‡	3.5	†	†
Ether-oxygen continued	4	47.0‡	18.2	5.2	0.94

* Only 3 blood obtained. † Bloods not obtained. ‡ Number too small.

necessary to prevent voluntary movements. The times for both respiratory and cardiac failure were noted. At the moment of respiratory failure, blood was obtained from the left femoral vein and its content of procaine and p-aminobenzoic acid determined by a modified Bratton-Marshall method for sulfonamides (12).

Oxygen. Ten dogs were treated in the same manner as the controls with the exception that they were allowed to breathe 95% oxygen at atmospheric pressure. A constant flow of oxygen was employed and rebreathing was not permitted. The oxygen was administered from the start of the procaine injection until respiratory failure, at which time the oxygen was discontinued.

Analeptics. Other groups of dogs received in addition to oxygen, intravenous injection of analeptics as soon as possible after complete cessation of all respiratory movements. The dose of these drugs varied and was given either as a single injection or in divided amounts at 30 second intervals. The total amounts of the drugs given, expressed as mg/kg were as follows: Nikethamide, 70 to 500; Metrazol, 25 to 150; Picrotoxin, 1 to 10; and Lobeline 0.4 to 0.9.

Artificial respiration. Series A. Three experiments were performed in which oxygen was administered under slight pressure. This procedure was the same as those with the oxygen experiments except that the oxygen was continued after respiratory movements had stopped. Series B. Three experiments were performed as under oxygen with the institution of artificial respiration at respiratory failure. In these experiments the procaine infusion was not stopped until the blood pressure had fallen almost to zero. Series C. Eight dogs were infused with procaine in the same manner as the controls. After the respiratory movements had stopped, and a blood sample had been taken, the blood pressure was carefully watched until it began to fall rapidly and the heart sounds became weak. At this point artificial respiration was started in an attempt to save the animal.

Results. A summary of the results obtained from the experiments with air, oxygen, and analeptics is given in Table I. In all experiments the heart continued to beat after respiratory movements ceased. The length of time that the heart continued to beat varied from 1 to 8 minutes, and all animals failed to recover. The individual values obtained for survival times and blood concentrations also showed considerable variation. In general the blood concentrations of both procaine and p-aminobenzoic acid were higher the longer the infusion was continued. These findings

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confirm our earlier reports(11,13,14). It is evident, however, from the standard errors of the means that there was a true difference between the groups breathing oxygen and the control group breathing air. The animals receiving oxygen required twice the amount of procaine to produce respiratory failure as did the controls. The length of time that the heart continued to beat after respiratory failure was also prolonged by oxygen. This beneficial effect of oxygen on the heart was much greater when the oxygen was continued under slight pressure until cardiac failure. It can also be seen from Table I that the addition of stimulant drugs was without effect, even though the dose of the drugs was large enough to produce twitching in at least half the cases.

In the experiments where artificial respiration was instituted at the time blood pressure began to fall rapidly, 6 of the 8 animals recovered after from 3 to 18 minutes of artificial respiration. Two animals failed to recover but here the artificial respiration was withheld for 3 and 4 minutes respectively, and the blood pressure had fallen almost to zero.

Further evidence that heart failure is, to a large extent, due to asphyxia was obtained from the three experiments (Table II) where artificial respiration was instituted as natural respiration failed and the procaine infusion was continued until heart failure. In 2 of the experiments the dogs received a continuous injection of 2 mg/kg/min procaine HCl. In the first dog respiratory movements ceased after 18 minutes with a blood procaine level of 1.5 mg %. The artificial respiration machine was then connected and the injection of procaine continued at the same rate for 6 hours, at which time the experiment was terminated. The terminal blood levels are shown in Table II. No reflexes could be evoked during this time and the only symptoms were intermittent muscular tremors. In the second dog, respirations ceased at 12 minutes. As the artificial respiration was started the dose of procaine was doubled. The heart did not stop until 2 hours and 33 minutes later. The blood concentrations were 4.2 mg % procaine and 1.7 mg % p-aminobenzoic acid at the time of respiratory arrest. A third dog was studied by the same experimental procedure with the difference that 4 mg/kg/min procaine was given until respiratory failure and then 8 mg/kg/min until the heart stopped. Respiratory movements ceased at 12 minutes and the heart continued to beat for another 43 minutes. The blood levels for procaine in the second and third dogs were more than twice as high as any fatal levels(11) seen in dogs not receiving artificial respiration (Table II).

TABLE II. Artificial Respiration on the Toxicity of Procaine.

Dose of Procaine HCl mg/kg/min.		Time in min.		Terminal blood levels mg %	
Until resp. failure	With art. resp.	Resp. failure	With art. resp.	Procaine	P.A.B.A.
2	2	18	360*	3.3	16.7
2	4	12	153	26.7	20.8
4	8	12	43	27.0	5.4

* Experiment terminated dog in good condition.

Conclusions. 1. The primary cause of death from intravenous procaine when used as an adjunct to general anesthesia is hypoxia due to respiratory failure. 2. If hypoxia is great at the time of respiratory failure, heart failure may occur almost simultaneously. 3. Nikethamide, Metrazol, Picrotoxin, and Lobeline are of no value in respiratory failure resulting from prolonged intravenous infusions of procaine. 4. Oxygen administration and artificial respiration are life saving if instituted promptly.

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Antagonism of Adrenocortical Extract and Cortisone to Desoxycorticosterone: Brain Excitability in Adrenalectomized Rats.* (18389)

Dixon M. Woodbury, John W. Emmett, George V. Hinckley, Newton R. Jackson, John D. Newton, John H. Bateman, Louis S. Goodman, and George Sayers.

From the Department of Pharmacology, University of Utah College of Medicine, Salt Lake City

It has been demonstrated that adrenocortical extract (ACE)(1) and cortisone(2) will counteract the depressive effect of desoxycorticosterone acetate (DCA) on brain excitability in intact rats. The present report concerns the effects of ACE and cortisone on the brain excitability of adrenalectomized DCA-implanted rats.

Methods. Adult male rats from the Sprague-Dawley farm were employed. The technic for determining the electroshock threshold (EST) has been presented in detail by Davenport(3). Alterations in EST observed throughout the course of the experiments have been expressed as the percentage change from the control value determined at the conclusion of a period of stabilization(4). Two to 4 days elapsed between each EST measurement. At the end of the stabilization period the animals were divided into 6 groups of 6 rats each and given the following treatments. The rats in Groups 1 to 4 were bilaterally adrenalectomized and implanted subcutaneously with six 15-mg pellets of DCA.† In addition, the animals in these groups received the following treatment: Group 1, a 0.9% sodium chloride solution to drink; Group 2, a 0.9% sodium chloride solution to drink and 0.5 ml of ACE‡ injected subcutaneously twice daily from the 21st to the 27th day of the experiment; Group 3, a 0.9% sodium chloride solution to drink and 1 mg of cortisone acetate (suspended in 0.2 ml of 10% ethanol) injected subcutaneously twice daily from the 21st to the 27th day; Group 4, water to drink plus 1 mg of cortisone acetate injected subcutaneously twice daily from the 33rd to the 36th day of the experiment. Groups 5 and 6 were composed of intact rats given water to drink. The animals in Group 5 were implanted with DCA and received no further treatment. The rats in Group 6 served as untreated controls.

Results. The results of these experiments are illustrated graphically in Fig. 1 and 2. The lines connect the average EST values of the rats in each group. It can be observed in Fig. 1 that the intact untreated rats in Group 6 showed a slight elevation in EST starting on the 22nd day and reached a value of about plus 5% on the 37th day. This elevation of EST is associated with an increase in weight of the animals and has been noted previously by Davenport and Davenport(5). The intact DCA-implanted rats in Group 5 showed an elevation in EST which exceeded that of the controls; the elevation was first observed on the 17th day and appeared to have reached a maximum of about 13% by the 32nd day. The EST of the intact DCA-implanted rats increased at a relatively slow rate as compared to previously reported experiments(1). The animals in Group 4, which were adrenalectomized, implanted with DCA and given water to drink, showed a more rapid rate of increase of EST

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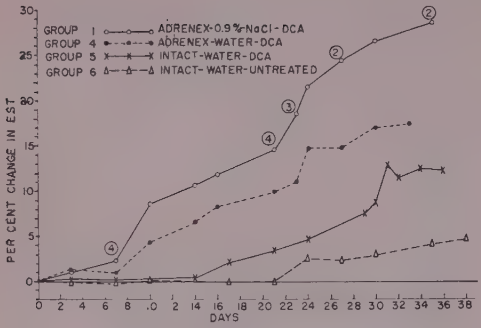

FIG. 1.

Effect of adrenalectomy, DCA and drinking fluid on EST. See text for explanation.

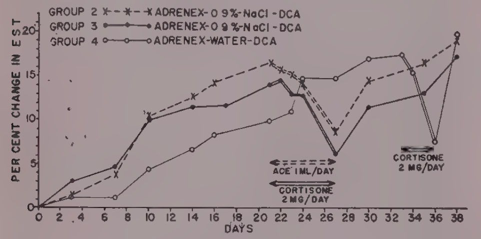

FIG. 2.

Effect of ACE and cortisone on the elevated EST of adrenalectomized DCA-implanted rats. See text for explanation.

than did those in Group 5. Furthermore, Group 4 reached a higher maximal elevation (17.5% after 33 days) than did Group 5. The EST of Group 1, the animals of which were adrenalectomized, implanted with DCA and given 0.9% sodium chloride solution to drink, rose rapidly and reached a value (plus 28.5% on the 35th day) much higher than those of the other groups. The figures in circles indicate the number of the original six animals which remained alive at the indicated times. No deaths occurred in the other groups.

Fig. 2 illustrates the data obtained on the effect of ACE and cortisone on the elevated EST of adrenalectomized DCA-implanted rats. Both ACE (Group 2) and cortisone (Group 3) decreased the elevated EST of DCA-implanted rats receiving 0.9% sodium chloride solution to drink; both substances, in the doses employed, induced about the same decrease (8.5%) in EST during the 6-day period of injection. It is of interest that these substances also induced loss in body weight; the decreases in weight during the period of administration were 9.5 and 9.1% for ACE and cortisone, respectively. Following withdrawal of the hormones the values of the EST slowly returned to levels slightly higher than those of the pre-injection period.

The adrenalectomized DCA-implanted rats, given water to drink (Group 4), showed a marked and rapid decrease in EST when treated with 2 mg of cortisone per day. The EST decreased 10% in 3 days as compared with 8.5% in 6 days for the animals on sodium chloride. The weight loss for this group during the injection period was 3.7% which is considerably less than that which occurred in the animals given sodium chloride solution to drink. It appears that the degree of reduction in EST is not related to the degree of loss of body weight. Following withdrawal of the cortisone the EST returned to a value greater than the preinjection threshold in 2 days as compared to 11 days for the animals on sodium chloride. Thus the EST of the DCA-treated rats on sodium chloride decreased less rapidly and recovered more slowly than did the EST of DCA-treated rats which received water to drink.

Discussion. The concept has been developed in this laboratory that DCA induces a state of hormone imbalance characterized by an excess of administered DCA and a deficiency of cortisone-like steroids of the adrenal cortex. Certain consequences of the deficiency can be overcome by stimulation of the adrenal cortex with ACTH or by the administration of ACE or cortisone. For example, in the intact rat it has been demonstrated (a) that DCA-induced insulin hypersensitivity can be counteracted by ACE administration(6), (b) that DCA-induced hypernatremia can be prevented by administration of ACTH or ACE (1), (c) that DCA-induced elevation in EST can be antagonized by ACTH, ACE or cortisone(1), and (d) that DCA-induced pathology (periarteritis nodosa, interstitial myocarditis and nephritis) can be markedly inhibited by ACTH or cortisone treatment(7). It has been suggested that the mechanism of

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action of DCA in producing a deficiency of cortisone-like steroids has two components: (1) inhibition of pituitary adrenocorticotrophic activity and (2) antagonism of DCA to cortisone-like steroids at their sites of action. Evidence in support of the first component is that ACTH counteracts certain effects of DCA and that DCA induces adrenocortical atrophy. [See review by Sayers(8)]. The data of the present report indicate that the second component is of major importance. Cortisone administration has been demonstrated to reduce EST in normal rats(4); in addition, the DCA-induced increase in EST in normal animals can be antagonized by cortisone(4). These facts alone could be interpreted to mean that cortisone affects brain excitability and that DCA exerts its effect on EST merely by inhibiting the pituitary release of ACTH, thus causing a cortisone deficit. However, it is herein shown that cortisone and ACE antagonize DCA in regard to brain excitability in the *adrenalectomized* animal in which the endogenous secretion of cortical steroids is nil; hence the antagonism is exerted in this instance not via the adenohypophysis but directly on brain excitability.

The nature of the antagonism between DCA and cortisone at their peripheral loci of action is unknown. The antagonism may be physiological, *i.e.*, the end result of two opposing metabolic actions which may or may not involve the same target tissue for each steroid. A more likely possibility is that DCA and cortisone, substances very similar in chemical structure, compete for the same strategic loci in the cell.

The fact that the EST of the adrenalectomized DCA-implanted animals given water to drink rose more rapidly than the EST of intact DCA-implanted rats given water to drink is additional evidence in support of the thesis that the secretion of the adrenal cortex opposes many of the actions of DCA. If DCA-implantation had completely inhibited ACTH release, one would not anticipate any difference between the response of adrenalectomized and intact rats to DCA treatment. However, such DCA treatment does not entirely suppress adrenocortical function. For example, adrenal atrophy is less marked in DCA-implanted animals than in hypophysectomized animals(1,9).

It is of interest that cortisone induced a more rapid decrease in EST in rats given water to drink than in rats given a solution of sodium chloride to drink and that the rate of increase of EST was greater in adrenalectomized rats given sodium chloride than in adrenalectomized rats given water to drink. It has been demonstrated that an important factor in determining brain excitability is the concentration of extracellular sodium; EST and the concentration of extracellular sodium parallel one another. One is tempted to interpret these results to mean that the rats loaded with sodium responded more slowly to cortisone because it took a longer period of time to reduce extracellular sodium in these animals. However, such an interpretation fails to explain the more rapid return of the EST to pre-injection levels in the rats given water to drink as compared to that in the rats given a solution of sodium chloride to drink. Electrolyte analyses may help to clarify this aspect of the problem.

The possibility that the DCA-cortisone antagonism on brain excitability may be used as a method of assay for cortisone-like compounds is suggested by the fact that cortisone and ACE gave similar responses in doses that have been found to be equivalent by other biological tests such as the Ingle-work test and the liver glycogen-deposition test(10). This type of assay is being explored by us.

Summary. The electroshock seizure thresholds (EST) of intact and adrenalectomized rats implanted with desoxycorticosterone acetate (DCA) and given water or a 0.9% sodium chloride solution to drink have been measured. The rate of elevation of EST was as follows, in increasing order: intact controls on water; intact DCA-implanted animals on water; adrenalectomized DCA-implanted rats

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on water; adrenalectomized DCA-implanted rats on 0.9% sodium chloride solution. These rates correspond to those predicted on the basis of the known effects of the secretion of the adrenal cortex and the concentration of extracellular sodium on brain excitability. Cortisone and adrenocortical extract (ACE) lowered the elevated EST of adrenalectomized DCA-implanted rats which were given a solution of sodium chloride to drink by 8.5% in 6 days. Following withdrawal of cortisone or ACE the EST returned to pretreatment levels in 11 days. Cortisone lowered the elevated EST of adrenalectomized DCA-implanted rats given water to drink by 10% in 3 days; following withdrawal of cortisone the EST returned to the pretreatment level in 2 days. It has been suggested that the results obtained provide evidence for the concept that cortisone and DCA compete for strategic loci in target cells.

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Hemagglutination by Col-MM-Virus. (18390)

SVEN GARD AND LEO HELLER. (Introduced by Albert B. Sabin.)
From the Department of Virus Research, Caroline Institute, Stockholm, Sweden.

Members of the EMC group of viruses(1,2) are capable of agglutinating sheep erythrocytes as first shown by Hallauer(3) and confirmed by Bremer and Mutsaars(4,5), Verlinde and de Baan(6) and Olitsky and Yager (7). Hemagglutinating capacity of the GD VII strain of Theiler's virus against human group O red blood cells was reported by Lahelle and Horsfall(8) and of Japanese B encephalitis virus for both sheep and chick erythrocytes by Sabin and Buescher(9). In all those cases, the hemagglutination could be inhibited by homologous immune sera.

Working with the Col-MM strain(10) of EMC virus, we have tried to establish a standard technic for assay of hemagglutination-inhibiting antibody for the purpose of studying the importance of this group of viruses as cause of disease in man(11-14). The difficulties encountered in virus hemagglutination (HA) experiments in general can be referred to the following complications: (a) the insensitivity of hemagglutination as a means of demonstrating the presence of virus, the hemagglutinating activity being inferior to infectivity by several powers of ten, (b) the appearance of false positives, caused by crude tissue extracts(7), bacterial contamination, so called spontaneous agglutination, etc. In order to increase the sensitivity of the HA reaction we have used very dilute cell suspensions [*cf.* Whitman(15)], the erythrocyte concentration ultimately adopted being 0.05%. Under such conditions the effects of salt agglutination and similar disturbances are more pronounced and the system has to be stabilized. This can be achieved by addi-

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tion of serum, albumin, or other proteins. We have found extracts of normal mouse or sheep brain, clarified by medium speed centrifugation, to be excellent stabilizers, superior to serum and bovine plasma albumin in giving more clear-cut negative patterns in the absence of virus and, thus, sharper endpoints, especially in hemagglutination-inhibition titrations.

In the experiments to be reported infected mouse brains served as source of virus and a 10% sheep brain extract, clarified by centrifugation for 30 minutes at 12000 G, was used as diluent. The red cells were stored in Alsever's or ACD I solution, washed and resuspended in veronal-buffered saline of pH 7.3-7.4. Salk's method of recording agglutination was used throughout.

Hemagglutination-inhibition (HI) by electrolytes. Inhibitors of the type described by Sabin and Buescher(9), effective against Col-MM hemagglutinin, were not found in extracts of normal or infected brain. Neither was hemagglutination inhibited by a number of pseudomucinous ovarian cyst fluids* of blood groups A and O, showing a high inhibitor activity against influenza virus hemagglutinin. Unlike the case of Japanese B virus(9) Col-MM virus hemagglutination proved to be independent of pH in the range of 6.0-9.0. On the other hand the ionic composition of the medium was of the utmost importance. Hypertonic salt solutions tended to suppress hemagglutination. As to inhibiting capacity, however, there was a pronounced difference between positive and negative ions. Whereas mono- and divalent cations showed no obvious effects, multivalent anions caused a considerable inhibition, roughly proportionate to their charge. The results recorded in Fig. 1 show, for instance, that hexametaphosphate in a concentration of 0.0002M inhibited completely 4 HA units of virus.

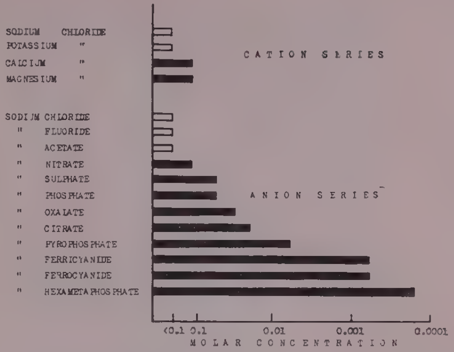

FIG. 1.

Inhibiting effect of different ions on Col-MM virus hemagglutination.

According to Whitman(15) the amount of specific immune serum needed for inhibition of influenza A virus is proportionate to the dose of virus, indicating the combination of virus and antibody as a principal factor in the mechanism of inhibition. In the experiment, summarized in Table I, inhibition by hexametaphosphate and immune serum was compared from this point of view. The HI titers of the 2 reagents were determined against 6 different virus doses and 4 different red cell suspensions. As shown in Table I there was an almost perfect rectilinear relationship between virus and inhibiting serum dose. Except for certain irregularities in systems with very dilute cell suspensions and large doses of virus, an increase in the concentration of virus used in the test caused an exactly corresponding decrease in HI-titer of the serum. On the other hand, the HI-titer of hexametaphosphate was largely independent of the amount of virus present. Thus a 32-fold decrease in virus concentration led to an 8-fold increase in HI-titer in a 0.2% cell-suspension and only 2-fold in 0.025% suspensions. The results indicate that hexametaphosphate acts upon the red cell rather than upon the virus. This interpretation received additional support in the results of virus neutralization experiments in which maximal non-toxic concentrations of Na-hexametaphosphate (0.1 ml of a 0.05-molar solution), substituted for serum in an otherwise classical intraperitoneal mouse neutralization test(16,17) failed to neutralize 10 and 50 LD_{50} respectively of Col-MM virus-amounts,

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* Obtained through the courtesy of Dr. R. Grubb, Bacteriol. Institute, University of Lund, Sweden.

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TABLE I. Hemagglutination-inhibition Titers of Sodium Hexametaphosphate and of Rabbit Col-MM-antiserum Titrated Against Varying Amounts of Col-MM Virus Hemagglutinin and of Sheep Red Cells.

Final conc. of red cells,%	Final dilutions of virus: 3:40	3:80	3:160	3:320	3:640	3:1280	3:40	3:80	3:160	3:320	3:640	3:1280	Increase in HI-titer corresponding to 32-fold decrease in virus: Na-HMP*	Serum
	Hemagglutination-inhibition titers of: Sodium hexametaphosphate (final molarity)						Col-MM antiserum (final dilution)							
.2	M/800	M/800	M/800	M/1600	M/3200	M/6400	1:64	1:128	1:256	1:512	1:1024	1:2048	8-fold	32-fold
.1	M/800	M/800	M/800	M/1600	M/1600	M/3200	1:64	1:128	1:256	1:512	1:1024	1:2048	4-fold	32-fold
.05	M/800	M/800	M/800	M/1600	M/1600	M/1600	1:64	1:128	1:256	1:512	1:1024	1:2048	2-fold	32-fold
.025	M/800	M/800	M/800	M/1600	M/1600	M/1600	1:32	1:64	1:256	1:512	1:1024	1:2048	2-fold	64-fold

* Na-HMP = Sodium hexametaphosphate.

by several powers of 10 smaller than those inhibited *in vitro* by c:a 100 times lower concentrations of the same salt.

The following experiment illustrated further the differences between the two types of inhibitors. In one set of tubes serial dilutions of the inhibitor were mixed with a fixed amount of virus. The mixtures were kept at +4°C for one hour, after which the red cell suspension was added. In another set the order of mixing was reversed, the virus being allowed to act upon the red cells for one hour before addition of the inhibitor. The results are presented in Table II. The table shows that the inhibiting effect of immune serum was considerably reduced once the combination between virus and cell had taken place. As a certain activity remained even under these conditions, either immune serum has the capacity to a certain extent to split the virus-cell combination, or else a partial dissociation of the latter is taking place in connection with the slight rise in temperature during manipulation. On the other hand, the effect of hexametaphosphate was exactly the same, irrespective of the order of mixing.

Following the same line of approach we tested also a crude preparation of thymus tetranucleotide [a product of controlled and specific enzymic hydrolysis of thymus desoxypentosenucleic acid(18)], chosen as being a more 'physiologic' polyvalent negative ion, and found it to be an exceedingly strong inhibitor, active down to the concentration of about 0.1 mg/ml. This corresponds to c:a 0.0001-molarity, assuming an average tetranucleotide size of the molecules of this no doubt heterogeneous oligonucleotide preparation. At the same time muscle adenylic acid (AMP)—a representative of the class of mononucleotides which, due to the very low second ionization constant of their phosphoric acid rest, must be assumed to appear in the neighborhood of pH 7 as prevalently monobasic—was found to be devoid of inhibiting activity at concentrations as high as 15 mg/ml (the highest tested), corresponding to c:a 0.05-molarity. Two crude preparations of

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TABLE II. Reversibility of the Col-MM Virus Hemagglutination by Homologous Immune Antisera and by Sodium Hexametaphosphate (Na-HMP).

Inhibitor	No. HA-units of virus	Initially mixed:	After 1 hr at +4°C added:	HI-titer	Difference in titer
Col-MM Antiserum	4	Virus + antiserum	RBC	1:1920	
	4	” + RBC	Antiserum	1:320	6-fold
Na-Hexametaphosphate (Na-HMP)	4	” + Na-HMP	RBC	1/3200-M	
	4	” + RBC	Na-HMP	1/3200-M	None

Volumes used: Col-MM virus, 0.2 ml; antiserum resp. Na-HMP-dilutions, 0.4 ml; 0.3% sheep red blood cells (RBC), 0.2 ml.

ribose and desoxypentose nucleic acids (the former a commercial yeast nucleic acid 'Boehringer' and the latter a low viscosity product prepared from calf spleen) both inhibited, the latter, however, about ten times more than the former (on an absolute concentration basis). These experiments must be repeated with better defined materials before any definite conclusions can be drawn.

Serum hemagglutination-inhibition. For elaboration of a standard HI-technic the following observations were important. In a study on the stability of virus and antiserum we found that the hemagglutinating titers of Col-MM virus suspensions (10% suspensions of infected mouse brain in 0.85% saline, clarified by 30 minutes spinning at 12,000 G), stored at –60°C, used to show a slight but rather consistent increase during the first few days. On further storage, however, the hemagglutinating capacity decreased continually to disappear completely after a period of several months. A parallel loss of infectivity was also demonstrable. Generally, virus suspensions older than 4-6 weeks could not be used for the HI-tests. Inactivation proceeded more rapidly on storage at higher temperatures as well as upon repeated thawing and freezing. A lyophilized rabbit hyperimmune serum showed a high and constant HI-titer when tested against freshly harvested virus hemagglutinin but gradually lower titers in tests with aging virus preparations. A plausible explanation of this phenomenon is the following. A loss of hemagglutinating activity and infectivity is not necessarily accompanied by a corresponding decrease in the capacity of combining with specific antibodies. One hemagglutinating unit of a partially inactivated virus comprises a larger quantity of virus protein than the same unit of a 100% active virus, and might therefore have a much larger antibody-combining power. Consequently, the HI-titer of a given serum is not a fixed quantity but dependent upon the amount and the state of the virus used in titration. Two different titrations can be compared only if they are performed simultaneously with the same virus preparation and dose. We have found it necessary, therefore, when examining unknown sera, to run simultaneously a titer on a known standard serum and refer the inhibiting activity of a serum to that of the standard rather than to give the individual titer values. The technic finally adopted was as follows.

Methods. Sheep red cells (RBC) were stored and treated as described above. The use of phosphate buffer was omitted on account of its inhibitory effect and veronal-saline substituted for it. A 10% sheep brain extract clarified by centrifugation at 12,000 G was used as diluent for the virus hemagglutinin. A standard dose of 4 HA units was chosen. Sera to be examined were inactivated for 30 minutes at 56°C and then absorbed in the cold with washed packed sheep red cells. Occasionally the absorption had to be repeated for removal of all hemagglutinins. The standard serum, a rabbit hyperimmune serum, was likewise inactivated at 56°C and absorbed with sheep red cells and normal mouse brain. It was lyophilized in exactly measured amounts of 0.2 ml, sealed off in a nitrogen atmosphere and stored at –15°C. Tests were set up with 0.2 ml of serial dilutions of serum, 0.1 ml of virus dilution, containing 4 HA units, and, after ½ hour at room

TABLE III. Results of HI-Titrations on Human Sera.

Group of patients	No. of sera tested	HI-index:	No. and (%) of sera within each group classified as: Negative <1	Weak 1-4	Positive 8	16	32	>32
1. Neurotropic infections (paralytic and non-paralytic poliomyelitis, aseptic meningitis, encephalitis, etc.)	384		281 (73.2%)	55 (14.3%)	29 —(48 (12.5%)—	10	7	2
2. Various diseases (no involvement of the central nervous system)	39		39 (100.0%)	—	—	—	—	—
3. Normal controls	146		129 (88.4%)	16 (10.9%)	1 (0.7%)	—	—	—

temperature, 0.1 ml of a 0.2% RBC suspension was added. The racks were placed in the refrigerator and readings according to Salk were made after 4 hours and again the next day. The following controls were included: (a) test serum + RBC (absence of serum hemagglutinins) (b) HA-titration of the virus used, and (c) HI-titer of the standard serum.

The result was expressed as inhibiting activity in per cent of that of the standard serum = *HI index*. Sera with indices below 1 were classified as negative, reactions in the range of 1 to 4 were considered as doubtful or weak, and values of 8 or above were recorded as positive.

Results. The material so far examined consisted of 384 sera from patients showing involvement of the central nervous system (paralytic and non-paralytic poliomyelitis, aseptic meningitis, encephalitis, etc.), 39 sera from patients with various diseases showing no such involvement, and a control group of 146 sera collected from blood donors and healthy pregnant women in a prenatal clinic.

The results can be summarized as follows (Table III). In the first group 73.2% were negative, 14.3% showed weak and 12.5% definitely positive reactions. In the second group only negative reactions were observed. In the third group, finally, there were 88.4% negative, 10.9% weak reactions and one out of 146 sera (0.7%) gave an index of 8.

The difference between the first group on one hand and the remaining groups on the other is highly significant, the probability of its being due to chance being less than 0.001.

A number of human sera with high HI-indices were tested for virus neutralizing capacity. In parallel tests rabbit hyperimmune serum diluted to give corresponding HI-indices was employed. Neutralization was performed intraperitoneally in mice against 15-50 MLD of virus. The rabbit serum gave complete neutralization, *whereas all human sera failed to neutralize.*

Discussion. Olitsky and Yager(7) reported that combination of virus and red cells occurred only in the cold and that complete dissociation of this combination took place when the temperature was raised to about + 20°C, without apparent changes in the properties of either virus or cells. This observation, which we can largely confirm, seems to indicate that mainly low energy bonds are involved in the reaction. The fact that polyvalent anions have a strong inhibiting effect points in the same direction. It seems probable that the ions do not act upon the virus itself but on the cell, in some way influencing the reactivity of the receptor groups.

The preliminary results of our experiments with organic compounds of the nucleotide-nucleic acid group could be interpreted as effects of organic anions, in accordance with the previous findings. The quantitative difference between thymus and yeast nucleic acid might be indicative of some additional, more specific factor but has to be confirmed with more well-defined products before any conclusions could be drawn.

Whatever the mechanisms of these phenomena may be, it is evident that the Col-MM virus hemagglutination is extremely sensitive to changes in the composition of the medium.

Therefore, appearance of serum inhibitors cannot be considered by itself as evidence of infection with a member of the EMC group of viruses. As a matter of fact, the failure of human sera to neutralize the virus seems to prove that the inhibiting capacity is not due to the presence of specific antibodies and, thus, to exclude definitely the assumption of EMC virus as an etiologic factor in the cases in question. The inhibitor found might still be an antibody, although heterologous, or it might be a non-specific substance appearing in the blood in connection with disturbances of the central nervous system. This question will be the subject of future experiments.

Summary. 1. Hemagglutination of sheep red blood cells by the Col-MM strain of the EMC virus was inhibited by polyvalent anions, the inhibitor titers (HI-titers) of the different negative ions being roughly proportionate to their net electrical charge. 2. Whereas the HI-titer of specific antibody was inversely proportionate to the amounts of virus, indicating a stoichiometric combination of virus and antibody, the HI-titers of sodium hexametaphosphate (6-valent anions) were only slightly influenced by the quantity of virus added. The order of mixing of the reactants was of great importance for the serum inhibition but without effect on the activity of hexametaphosphate. 3. The strong hemagglutination-inhibiting effect of sodium hexametaphosphate was not paralleled by any virus neutralizing activity. 4. A standard serological HI-test, based on the results of a study of the Col-MM virus hemagglutination, is described. Applied on 569 human sera, this method gave a high incidence (12.5%) of strongly positive hemagglutination-inhibition titers among 384 sera obtained from patients showing distinct involvement of the central nervous system. Weakly positive reactions were encountered in 14.3%. A control group, consisting of 146 sera collected from healthy humans showed only 0.7% strong and 11% weak reactions, while 39 sera from patients with various ailments but apparently free from neurotropic infections were negative throughout. 5. Even strongly inhibiting human sera failed to neutralize Col-MM virus in animal experiments.

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Effect of Various Levels of Vitamin B_{12} Upon Growth Response Produced by Aureomycin in Chicks. (18391)

E. L. R. STOKSTAD AND T. H. JUKES.

From the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

It was reported by Stokstad and coworkers(1) that the growth of vit. B_{12}-deficient chicks was increased by the addition of a crude supplement prepared from fermentation with *Streptomyces aureofaciens*. The presence of vit. B_{12} and B_{12b} in this fermentation was subsequently demonstrated (2) and it was shown that the supplement also contained aureomycin which was found to increase the growth rate of chicks on diets either adequate(3,4) or deficient(5) in vitamin B_{12}. It thus became evident that either aureomycin or vit. B_{12} would produce a growth response in chicks on an all-vegetable diet and some evidence was obtained(5) which suggested that aureomycin had a spar-

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ing action on the requirements of the chick for vit. B_{12}. It seemed of interest to investigate more fully the effect of aureomycin on the vit. B_{12} requirement of the chick.

Methods. Chicks (Barred Rock x New Hampshire) were obtained from eggs laid by hens on a corn-soybean-alfalfa diet as described previously(1) and were fed the experimental diets immediately. The chicks were markedly deficient in vit. B_{12}, but the severity of the deficiency varied from month to month as indicated by variations in rate of growth of chicks on the basal diets. Such variations are probably reflections of differences in the extent of carry-over of vit. B_{12} from the hen through the egg to the chick. This carry-over may change although the diet fed to the hens is kept constant, for it has been shown that vit. B_{12}-like factors are produced by microorganisms which are present in the feces(6) and coprophagy by the hens may be presumed to occur. The production of such factors in the litter of the henhouse is apparently influenced by the environmental temperature(7). Basal diets 1 (30% soybean meal) and 2 (70% soybean meal) which were supplemented with minerals, 0.3 DL-methionine and vitamins including 0.2% choline chloride have been previously described(3). Aureomycin HCl was recrystallized either twice or thrice before use.* Its vit. B_{12} content was found to be less than 0.2 μg per g by microbiological assay with *Lactobacillus leichmannii.*† An assay of a sample with *Euglena gracilis* indicated that the content was 0.1 μg per g according to a communication from Dr. W. J. Robbins. Solutions of vit. B_{12} for addition to the diets were prepared from crystalline compound and were standardized spectrophotometrically at 261 mu, $E^{1\%}_{1\,cm} = 208$.

TABLE I. Effect of Aureomycin on Growth at Different Levels of Vitamin B_{12}.

	% increase in growth* due to 100 mg aureomycin per kg diet at various levels of vit. B_{12}						
Exp. No.	0 μg B_{12}	3 μg B_{12}	5 μg B_{12}	10 μg B_{12}	20 μg B_{12}	50 μg B_{12}	Evidence of B_{12}-sparing action
1	−1			18	13	23	No
2	2		37	17		9	Yes
3	34		18	11	11		Yes
4	6	−4	12	11	17	15	No
5	10				9		No

* Weighed at 25 days. Diet 2 (70% soybean meal) was used in Exp. 1, 2, 3, and 4, and diet 1 (30% soybean meal) in Exp. 5.

Results. Various experiments were made in which graded levels of vit. B_{12} were fed to chicks in the presence and absence of aureomycin. The results, summarized in Table I, show the growth increases produced by aureomycin at different levels of vit. B_{12}. A sparing action of aureomycin on vit. B_{12} was noted in some experiments, such as Exp. 2, but not in others. In Exp. 6, Table II, 10 μg of vit. B_{12} in the presence of aureomycin gave almost the maximum response obtained with the vitamin but in the absence of aureomycin, 50 μg of vit. B_{12} per kilogram of diet produced a markedly greater response than 10 μg.

Aureomycin often produced little or no growth response but markedly decreased mortality in chicks when on the basal vit. B_{12}-deficient diet or when inadequate levels of vit. B_{12} were added. The chicks at hatching were markedly deficient in vit. B_{12} due to the fact that they were obtained from eggs laid by hens which were on a diet deficient in this vitamin. A high mortality of such chicks has been noted when they are placed on diets deficient in vit. B_{12}(1,8). A summary of all experiments pertaining to the effect of aureomycin on the mortality of vit. B_{12}-deficient chicks is presented in Table III. It shows that in every case the mortality was lower when aureomycin was added. Two explanations are possible for this reduction of mortality. First, aureomycin may reduce the requirement of the chicks for vit. B_{12}. Sec-

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* Prepared by the Chemical Production Section, Lederle Laboratories.

† Carried out by Dr. H. P. Broquist.

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TABLE II. Effect of Aureomycin on Growth of Chicks on Diet 2 at Various Dietary Levels of Vit. B_{12}.
Basal diet 1 (70% soybean meal) 16 chicks per group.

Exp. No.	Vit. B_{12} per kg diet, μg	Body wt and survivors ( ) at 25 days* No aureomycin, g	Aureomycin added, g†	% increase in wt due to aureomycin†
6	0	120 (6) ± 11‡	132(10) ± 11	10
	2	120 (6) ± 9	190(14) ± 12	58
	4	201(13) ± 18	240(14) ± 15	19
	5	203(14) ± 20	260(15) ± 17	28
	10	227(13) ± 20	337(16) ± 16	44
	50	331(15) ± 10	357(16) ± 14	8
7	0	164(15) ± 14	185(16) ± 12	13
	2	220(16) ± 9	271(16) ± 11	23
	4	237(15) ± 14	284(16) ± 12	20
	10	266(15) ± 8	273(15) ± 12	3
	50	294(15) ± 15	300(16) ± 12	2
8	0	125 (2) ± 0	129(10) ± 11	3
	3	146 (7) ± 19	197(15) ± 17	35
	5	200(10) ± 23	234(15) ± 18	17
	10	253(15) ± 18	309(16) ± 16	22
	20	303(16) ± 14	347(16) ± 10	14

* Average of 2 groups of 8 chicks each.

† Exp. 6 and 7, 100 mg of aureomycin; Exp. 8, 50 mg of aureomycin added per kilo of diet.

‡ Standard error = $\sqrt{\frac{\Sigma d^2}{n}} / \sqrt{n}$ where "d" = deviation from mean and "n" = number of observations.

ond, the deficient chicks may have a higher susceptibility than normal chicks to the deleterious effect of certain microorganisms in the intestinal tract, and aureomycin may decrease the number of these organisms.

The effect of another bacteriostatic substance, sulfamethazine, on the requirement of chicks for vit. B_{12} was investigated and the results are shown in Table IV. No sparing effect of sulfamethazine on the requirement for vit. B_{12} was found nor was a growth-promoting effect noted for sulfamethazine.

TABLE III. Effect of Aureomycin on Mortality at Low Levels of Vit. B_{12}.

Exp. No.	Vit. B_{12} per kg diet, μg	Mortality at 21 days No aureomycin, %	100 mg aureomycin per kg diet, %
1	0	25	8
2	0	58	8
	5	58	8
3	0	7	0
4	0	67	16
	5	41	8
5	0	50	31
	2	56	12
6*	0	87	25
	3	50	0
7	0	6	0
8	0	87	37
	3	56	6
	Avg	50	12

* 50 mg aureomycin added per kg diet in this experiment.

Discussion. It was shown by various investigators in studies with purified diets that the addition of bacteriostatic substances such as sulfaguanidine and succinylsulfathiazole produced a deficiency in the rat for certain water-soluble vitamins such as biotin and pteroylglutamic acid. This is usually interpreted as indicating that rats on purified diets are dependent upon their intestinal bacteria as a source of certain vitamins of the B complex. The present observations with chicks may indicate that under certain circumstances the use of an antibacterial substance may serve to increase rather than to decrease the supply of water-soluble vitamins. Moore *et al.*(9) reported that at marginal levels of pteroylglutamic acid the addition of succinylsulfathiazole produced a depression of

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TABLE IV. Effect of Sulfamethazine on the Response to Vitamin B_{12}. Basal diet 1 (70% soybean meal) 12 chicks per group.

	Body wt and survivors at 21 to 22 days				
	No sulfamethazine		0.5% sulfamethazine		
Vit. B_{12} per kg diet, μg	Individual groups, g	Avg, g	Individual groups, g	Avg, g	Response to sulfamethazine, %
0	116 (5) 91 (4)	103	117 (3) —	117	14
3	146 (8) 137 (5)	142	124 (4) 144 (9)	138	–4
5	159 (9) 194(11)	177	169(10) 148(10)	159	–10
10	188(12) 220(12)	204	221(11) 195(12)	208	2
20	230(12) 223(12)	227	225(10) 218(11)	222	–2
50	219(12) 245(11)	232	243(12) 227(11)	235	1

growth which may have been due to a decrease in the amount of pteroylglutamic acid which was synthesized by the intestinal bacteria. However, in the presence of an excess of dietary pteroylglutamic acid, the addition of succinylsulfathiazole produced an increase in the rate of growth, perhaps due to the suppression of certain deleterious intestinal bacteria. The effect of succinylsulfathiazole in depressing growth at marginal levels of pteroylglutamic acid may be contrasted with the effect of aureomycin in improving growth at marginal levels of vit. B_{12} in chicks in some of the present experiments. The effect of aureomycin in producing a hematological response in patients with pernicious anemia as noted by Lichtman and co-workers(10) may be compared with the effect of aureomycin in producing a growth response in chicks which were receiving an insufficient dietary supply of vitamin B_{12}. The oral administration of high levels of streptomycin to rats was reported by Smith and Robinson(11) to decrease the number of *E. coli* organisms in the digestive tract and to produce a simultaneous increase in *Bacillus megatherium*, the organism which was shown by Lewis and co-workers(12) to produce large quantities of vit. B_{12} by fermentation.

The presence or absence of a vit. B_{12}-sparing effect for aureomycin as shown by the variation in results in different experiments may be due to differences in the intestinal flora encountered. It would seem probable that variability may be expected when the growth response to a substance depends on the nature of the intestinal flora which are present in the test animal.

Summary. 1. The vit. B_{12} requirement for growth of chicks was studied on a deficient diet with and without the addition of aureomycin. In some experiments, a "sparing effect" of aureomycin upon vit. B_{12} requirement was noted but in others no effect was observed. A growth-promoting effect for aureomycin was noted both in the presence and absence of added vit. B_{12}. 2. The mortality of deficient chicks on the diet containing no added vit. B_{12} was markedly reduced by aureomycin. 3. The addition of sulfamethazine to the diet did not appear to effect the quantitative requirement of chicks for vit. B_{12}.

The assistance of Mr. V. Stryeski is gratefully acknowledged.

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Influence of Adrenaline and Insulin on Adrenal Cortical Response to ACTH. (18392)

Claude Fortier,* Floyd R. Skelton, and Paris Constantinides.
(Introduced by H. Selye.)

From Institut de Médecine et de Chirurgie Expérimentales, Université de Montréal, Canada.

Previous observations have shown that the oral administration of glucose to hypophysectomized animals markedly enhances the adrenal response to exogenous ACTH(1,2). The possibility was considered that the hyperglycemic level, *per se,* was responsible for the observed effects (increased adrenal cholesterol depletion, liver-glycogen deposition and thymus involution). In order to evaluate this possibility, the adrenal cortical response to ACTH was studied in hypophysectomized animals under conditions of adrenaline-induced hyperglycemia and insulinic hypoglycemia.

Materials and Methods. 56 male piebald rats of an average weight of 180 g were equally divided into 8 groups and the following general procedure adopted. Armour's ACTH (lot No. 60-61) was used at a dose level of 2 mg, adrenaline (epinephrine HCL) of 0.1 mg, and insulin (Toronto) of 0.5 unit per 100 g. ACTH was subcutaneously injected into hypophysectomized fasted rats. Adrenaline and insulin were administered by the same route, 60 and 90 minutes respectively after ACTH administration, and the animals were killed at intervals of 3 and 6 hours following the latter injection. Hypophysectomized control rats without further treatment or receiving ACTH, adrenaline or insulin alone were killed at corresponding time intervals. In this way, the full action of ACTH on adrenal cholesterol coincided with the maximal hyper- and hypoglycemic levels and, it was possible to study all events as a function of time. The time schedule was arranged in such a way that all animals were killed 24 hours after hypophysectomy and the beginning of fasting. Samples were removed for the following determinations: blood glucose by the method of Folin(3), adrenal cholesterol by the method of Sperry(4), adrenal and plasma ascorbic acid by the method of Roe and Kuether(5), and liver glycogen according to Venning's modification of the procedure described by Good, Kramer and Somogyi(6).

Results. The results are summarized in Table I.

Blood-sugar — ACTH alone was without effect on the blood sugar and did not significantly alter the adrenaline hyperglycemia or the hypoglycemic effect of insulin.

Plasma ascorbic acid — ACTH alone caused a significant although slight and transient elevation of the plasma ascorbic acid level, while a marked drop resulted from the administration of adrenaline and insulin either alone or in association with ACTH. The observed fall is apparently related to an increased utilization. It is in contrast, in the case of adrenaline hyperglycemia, with the marked potentiation of the ACTH-induced rise in the plasma ascorbic acid concentration observed in previous experiments to follow alimentary hyperglycemia(2,7).

Liver glycogen—A slight deposition of glycogen was evidenced in both adrenaline

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* This work was done during the tenure of an American Heart Association research fellowship.

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TABLE I. Effects of Adrenaline and Insulin on Response of ACTH.

Chemical determinations	Blood sugar, mg/100 ml			Plasma ascorbic acid, γ/100 cc			Liver glycogen, g/100 g			Adrenal cholesterol, mg/100 mg			Adrenal ascorbic acid, γ/100 mg		
Time intervals*	0	3 hr	6 hr	0	3 hr	6 hr	0	3 hr	6 hr	0	3 hr	6 hr	0	3 hr	6 hr
Treatment															
Hypophysectomy + ACTH†	95.2 ±4.9	100.4 ± 2.9	86.8 ± 8.6	1383 ±80	1580 ±14	760 ±60	T.‖	T.‖	T.‖	4.58 ±.18	3.17 ±.43	4.24 ±.36	540 ±19	417 ±27	488 ±17
Hypophysectomy + adrenaline‡	95.2 ±4.9	157.4 ±19.0		1383 ±80	1000 ±55		T.‖	.033 ±.036		4.58 ±.18	4.15 ±.29		540 ±19	565 ±12	
Hypophysectomy + insulin§	95.2 ±4.9	28.7 ± 2.4		1383 ±80	967 ±118		T.‖			4.58 ±.18	4.05 ±1.2		540 ±19	562 ±17	
Hypophysectomy + ACTH + adrenaline	95.2 ±4.9	199.6 ±33.2	85.3 ±18	1383 ±80	780 ±80	960 ±40	T.‖	.007 ±.003	T.‖	4.58 ±.18	3.56 ±.45	3.89 ±.16	540 ±19	416 ± 5	452 ±37
Hypophysectomy + ACTH + insulin	95.2 ±4.9	27.0 ± 3.8	27.0 ± 8.0	1383 ±80	1020 ±73	900	T.‖	T.‖	T.‖	4.58 ±.18	4.25 ±.41	3.62 ±.31	540 ±19	416 ±11	404 ±49

* Time expressed in hr after ACTH administration. In groups not receiving ACTH, the animals were killed 2 hr after adrenaline and 1½ hr after insulin administration.

† ACTH, Armour, Lot No. 60.61, 2 mg/100 g.

‡ Adrenaline HCL. 0.1 mg/100 g.

§ Insulin, Toronto, 0.5 U./100 g.

‖ Traces.

treated groups, while traces only were detectable in the others.

Adrenal cholesterol and ascorbic acid—30 and 20% falls were respectively observed in the concentrations of cholesterol and ascorbic acid 3 hours after the administration of ACTH. Adrenaline and insulin alone were without effect on these adrenal constituents, neither did they alter significantly, when associated with ACTH, their response to corticotrophic stimulation.

Conclusions. The above results show that adrenaline or insulin induced alterations of the blood sugar level do not modify the adrenal response to ACTH. The previously noted effect of glucose administration on the adrenal cholesterol response to ACTH is therefore attributed to an increase in the total store of carbohydrates or to an excess production of intermediary metabolites.

Summary. In order to investigate the mechanism of the previously observed enhancement of the adrenal response to ACTH following the administration of glucose, this response was studied in hypophysectomized animals under conditions of adrenaline hyperglycemia and insulin-induced hypoglycemia. Determinations were made of blood-sugar, plasma ascorbic acid, liver glycogen, adrenal cholesterol and ascorbic acid.

1. The blood sugar changes resulting from adrenaline or insulin administration were not influenced by ACTH. 2. A marked fall in plasma ascorbic acid concentration resulted from the administration of adrenaline and insulin either alone or in association with ACTH. 3. Neither adrenaline nor insulin modified the response to ACTH of liver glycogen and of adrenal cholesterol and ascorbic acid. 4. The effects of alimentary hyperglycemia are therefore attributed to an increase in the total store of carbohydrates or to an excess production of intermediary metabolites, which conditions are not duplicated by adrenaline.

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Effect of Aureomycin on Viruses of the Psittacosis-LGV Group in Embryonated Eggs and Mice. (18393)

JOHN C. WAGNER, VIVIAN W. ANDREW, AND MARY S. WATSON.
From the Biological Department, Chemical Corps, Camp Detrick, Frederick, Md.

The efficacy of aureomycin(1) in the treatment of bacterial and viral diseases made it desirable to obtain additional data on the effect of this antibiotic on viruses of psittacosis-LGV group.

Preliminary reports by Wong and Cox(1) showed that aureomycin, in mice and eggs infected with some strains of the psittacosis-LGV group, was effective in counteracting the lethality of the virus. Considerable significance therefore has been attached to the antiviral activity of this drug. Should the drug show similar activity for other psittacosis-LGV strains, a useful adjunct or substitute for either penicillin or sulfadiazine therapy (2) would be indicated. This paper will present the results of such a study.

Materials and Methods. Virus Strains. Ten representative strains from both mammalian and avian sources were included in the studies. The 6BC, Borg, Gleason, S-F and P-207 viruses were employed in animal studies, while the Cal 10, LGV, 12xN, Baker and Nigg viruses were included in the egg experiments. The source of these strains has been presented previously(2).

Egg Studies. Groups of fertile eggs, 8 or 9 days old, were inoculated by the yolk sac route with (1) 0.25 ml of a M/15 phosphate buffer solution (pH 7.0), or (2) 0.25 ml of buffer solution containing 0.5 mg of aureomycin per egg. Two to 4 hours later both groups of eggs were injected by the yolk-sac technique with 0.25 ml of 10-fold broth dilutions of the viral strain under test, using 20 eggs for each dilution. Yolk sac material was employed as the source of virus in all tests. The eggs were candled daily for 10 days and deaths recorded. At intervals the yolk sacs of dead eggs were examined microscopically by the Macchiavello technique for virus. The effect of the drug on the infection was evaluated by comparing the titers of the virus in LD_{50}(3), in the 2 series.

Animal Studies. Groups of 80 or more white mice, weighing 15-18 g were inoculated intracerebrally with 0.03 ml of virus suspension in nutrient broth representing (1) 50 mouse intracerebral LD_{50} or (2) 1000 mouse intracerebral LD_{50}. The groups were then subdivided into lots of approximately 15 mice each for treatment with aureomycin which was started 48 hours following virus inoculation and continued for 5 consecutive days. Daily subcutaneous inoculations of 0.25 ml of aureomycin solution were given representing 0.5 mg, 0.1 mg, or 0.02 mg of drug and the test observed for 30 days unless otherwise noted. Control groups of animals received either virus or drug alone. In some experiments the treatment was repeated at 9 and 19 day intervals following virus inoculation to provide more extensive treatment.

Concomitant studies were undertaken to determine the concentration of aureomycin in blood, urine and tissues of mice receiving aureomycin treatment as described. Approximately 24 hours after each aureomycin injection, 3 mice were sacrificed, the organs ground separately in sterile distilled water and the tissue suspensions analyzed by an inhibition technique(4).* The carrier state of drug treated animals in each group was checked at various times. Three mice were sacrificed, the brains removed aseptically and 10% emulsions prepared in sterile nutrient broth. The suspensions were centrifuged at 1000 rpm for 5 minutes and the supernatant fluids re-

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* The lowest range of sensitivity of the test is considered to be 0.3 μg of drug per ml of fluid.

TABLE I. Effect of Aureomycin on Growth of 6BC Virus in Eggs.

	Results in untreated eggs	Results in treated eggs*		
Dilution of viral inoculum	Survival ratio†	Survival ratio‡	Yolk sac smear of surviving eggs	LD_{50} titer of surviving eggs
10^{-2}	0/16†	9/14	+	$10^{-8,4}$
10^{-3}	0/16	15/20	+	$10^{-8.3}$
10^{-4}	0/18	14/16	+	$10^{-8.3}$
10^{-5}	0/18	15/17	+	$10^{-6.9}$
10^{-6}	0/18	17/20	—	$10^{-4.0}$
10^{-7}	0/18	16/20	—	$10^{-3.8}$
10^{-8}	0/18	15/17	—	$10^{-2.0}$
10^{-9}	8/14	13/13	—	$10^{-2.0}$

* A single yolk-sac inj. of 0.5 mg of drug 2-3 hr following virus inoculation.

† LD_{50} endpoint of control titration $10^{-8.8}$.

‡ LD_{50} endpoint of test titration $10^{-2,8}$.

§ This calculation, although not statistically valid, represents a rough estimation of the endpoint.

inoculated intracerebrally into groups of 10 normal mice. The animals were observed for 21 days and the deaths recorded. Irregular deaths were checked for virus by microscopic examination of brain tissues.

Experimental. Experiments with Eggs. Table I shows results obtained when aureomycin was tested against the 6BC strain. The drug was extremely effective in controlling the infection as evidenced by comparing the titer of the virus in drug treated embryos (LD_{50} $10^{-2.8}$) with the titer of the same virus in untreated control embryos (LD_{50} $10^{-8.8}$). This indicates a neutralization of at least 1.0 x $10^{6.0}$ LD_{50}'s of virus. Since it was evident that a considerable number of eggs would survive in all of the drug treated groups, it was desirable to determine whether the virus inoculated into these embryos was killed by the drug or merely inhibited in its growth. At the end of the observation period 2 or more eggs were selected at random from among the surviving drug treated eggs in each virus dilution group. The yolk sacs from these eggs were pooled and titrated for virus by inoculation of serial dilutions into eggs by the yolk sac route. The results of these tests are also shown in Table I.

Yolk sacs of treated eggs surviving infection following inoculation with 6BC virus dilutions $10^{-2.0}$, $10^{-3.0}$ and $10^{-4.0}$ yielded virus titers of $10^{-8.4}$, $10^{-8.3}$ and $10^{-8.3}$ respectively. These titers approximate those seen in heavily infected yolk sacs, *e. g.*, control titration ($10^{-8.8}$) in Table I. As the amount of virus inoculated into the drug treated embryos decreased (dilutions of $10^{-5.0}$, $10^{-6.0}$ and $10^{-7.0}$) the titer of virus in the surviving eggs correspondingly decreased to $10^{-6.9}$, $10^{-4.0}$ and $10^{-3.8}$ respectively. In eggs given virus dilution $10^{-8.0}$ none of the control embryos survived while 15 of 17 drug treated eggs lived, with a subsequent yolk sac passage titer of less than $10^{-2.0}$.

Microscopic examination of stained yolk sac smears of surviving drug treated eggs showed elementary bodies in groups inoculated with virus dilutions $10^{-2.0}$ through $10^{-5.0}$, but not in groups receiving dilutions $10^{-6.0}$ or greater. It can be seen (Table I) that, at the dilution point between positive and negative smears, the titer of virus in surviving eggs dropped from $10^{-6.9}$ to $10^{-4.0}$. These data point out the advisability of employing titration methods rather than microscopic examination for evaluating drug efficacy in embryos.

The consolidated results of similar data for all 10 virus strains in aureomycin treated embryos are shown in Table II. All the virus strains followed a pattern similar to that described in detail for the 6BC strain. Only in those strains usually considered to be most infectious; *i.e.*, 6BC, Borg, Gleason, S-F, P-207 and Cal 10, was it possible to demonstrate high titer virus in surviving drug treated embryos. Correspondingly LGV, 12xN, Baker, and Nigg strains in most instances yielded virus titers so low as to suggest complete inhibition of growth in all dilutions tested.

Experiments with Mice. The results of these studies are presented in Table III. As was expected, not all of the virus strains had the same degree of susceptibility to the drug. In general, however, all were affected by 0.5 mg of aureomycin administered over a 5 day period. This is evident by the extended average day of death (A.D.D.) of treated ani-

TABLE II. Effect of Aureomycin on Growth of Psittacosis Strains in Eggs.

	Titer of virus LD_{50} (negative log)		LD_{50} (negative log) titer of drug treated eggs Surviving on dilutions							
Virus strain	Untreated eggs (control)	Drug treated eggs	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
6BC	8.8	2.8	8.4	8.3	8.3	6.9	4	3.8	<2	<2
Borg	8.5	3.7	8.5	8.5	8.5	8.4	8	3.5	3.5	1
Gleason	8.0	2.4	8.3	8.3	8.3	4.6	<2	<2	<2	<2
S-F	6.7	<2	>8.5	>8.5	8.2	4.6	4.9	<2	<2	
P-207	7.3	2.4	>7.5	7.5	3.9	3.9	<2	<2	<2	
Cal 10	7.1	<2	>8.5	8	6	6	<2	<2	<2	<2
LGV	3.5	<2	3.4	<2	<2	<2				
12xN	5.4	<2	<2	<2	<2	<2				
Baker	5.66	<2	<2	<2	<2	<2	<2			
Nigg	5.2	<2	<2	<2	<2	<2				

TABLE III. Effect of Aureomycin in Mice Inoculated Intracerebrally with Psittacosis Virus Strains.

		Survival ratio of mice After 5 day drug treatment with:							
		0.5 mg/dose		0.1 mg/dose		0.02 mg/dose		No treatment	
Strain	No. intra-cerebral LD_{50} inoculated	Surv./total	A.D.D.*	Surv./total	A.D.D.	Surv./total	A.D.D.	Surv./total	A.D.D.
6BC	50	4/12	15	0/14	13	0/12	6	0/12	5
	1000	7/12	14	0/13	9	0/12	5	0/12	5
Borg	50	12/12	>30	4/13	9	0/12	5	0/12	6
	1000	10/12	25	5/15	9	1/12	4	0/12	4
Gleason	50	6/12	13	0/13	7	0/12	5	0/12	5
	1000	8/12	17	0/14	9	0/10	4	0/12	5
S-F	50	20/20	>30	19/20	>22	8/20	8	0/34	7
	1000	9/20	23	15/20	19	2/20	8	0/31	7
P-207	50	2/12	15	0/13	12	0/12	5	0/12	6
	1000	10/12	25	0/12	18	0/11	4	0/12	5

* A.D.D.—Average day of death of animals dying on each drug treatment level.

mals as compared to untreated. The Borg and S-F strains appeared particularly susceptible, while the Gleason, 6BC and P-207 strains, although not showing a particularly impressive survival ratio for the 0.5 mg series, did show a marked extension in the average day of death (A.D.D.). In none of the tests did a dosage of 0.02 mg of drug appreciably alter the course of the disease. Fig. 1 and 2 present graphically the results observed when the 3 doses of aureomycin were tested against the Borg strain. Similar results were obtained for the other viruses.

It was observed that mice given aureomycin treatment and surviving infection with strain 6BC continued to harbor virus in brain tissues examined 21 days after inoculation. The development of a similar carrier state in animals infected with viruses of this group has been observed frequently in therapy studies employing such compounds as sulfadiazine, penicillin and chloromycetin. The results of testing all groups of surviving mice at the end of 30 days for evidence of living virus indicated that a carrier state was established in aureomycin treated animals inoculated with the 6BC, Borg, Gleason, S-F and P-207 virus strains, living virus being recovered from all groups of treated mice. There was no evidence to suggest strain differences from the results of these carrier studies. The action of aureomycin therefore appears to be similar to penicillin, chloromycetin and other chemotherapeutic compounds effective against viruses of the psittacosis-LGV group.

Distribution of Drug in Mice. Tests were conducted to determine the distribution of aureomycin in the tissues of normal mice following a series of drug treatments similar to those employed in the therapeutic studies. The results of these determinations are presented in Table IV. Aureomycin levels are

TABLE IV. Distribution of Aureomycin in Animal Tissues Following Repeated Subcutaneous 0.5 mg Injections of Drug.

No. of days of drug treatment	Results of assays of*					
	Blood†	Liver‡	Spleen	Kidney	Lung	Brain
1	0.3	7.5	TR§	5.0	TR	0
2	TR	7.6	0	7.7	0	0
3	0	4.6	0	TR	0	0
4	0	3.4	0	1.0	TR	0
5	TR	TR	TR	7.1	TR	0

* Values expressed as average of 3 individual samples.

† Blood level expressed as μg/ml.

‡ Tissue levels expressed as μg/g.

§ Trace quantities 0.3 μg/g.

expressed as the average of 3 individual samples. Only in liver and kidney were significant levels of aureomycin consistently detected. Blood samples following one day treatment yielded levels of approximately 0.3 μg/ml, with traces to negative levels thereafter. Spleen, lung and brain samples were essentially negative in all tests.† These results are particularly significant since, in the therapy studies, mice were infected by the intracerebral route and were benefited by drug treatments. There was no evidence for cumulative drug levels in any of the animal tissues when treated and sampled at 24 hour intervals.

Multiple Five Day Treatment Periods. Fig. 3 shows the survival ratio of 3 groups of 20 or more animals, all infected intracerebrally with 1000 mouse IC LD_{50} of 6BC virus, and each treated with drug. Group 1 received daily intraperitoneal injections of 0.5 mg of aureomycin beginning on the second day following virus infection and terminating 5 days later. Group 2 received the above schedule plus an additional 5 days treatment beginning on the ninth and extending to the thirteenth day. Group 3 received both the above schedules plus an additional 5 days treatment beginning on the nineteenth day and extending to the twenty-fourth day. It can be seen that the percentage of mice surviving in any group was directly influenced by the number of drug treatments. While only 30% of mice lived following the first treatment, the survival index was raised to 65% and 91%, respectively, by the second and third schedules. It should be noted, too, that in each of the 3 groups there was 6 to 9 days lapse between the cessation of treatment and the beginning of deaths.

Accompanying groups of mice were checked periodically to determine how long the virus remained viable in the brain following drug treatment. The brains of three mice from each group were removed, each ground to make a 10% emulsion in nutrient broth and reinoculated intracerebrally into 10 mice which were observed for 21 days. In 2 of the 3 groups, living virus was recovered 69 days following inoculation, while after 83 days no virus was recovered from any of the groups.

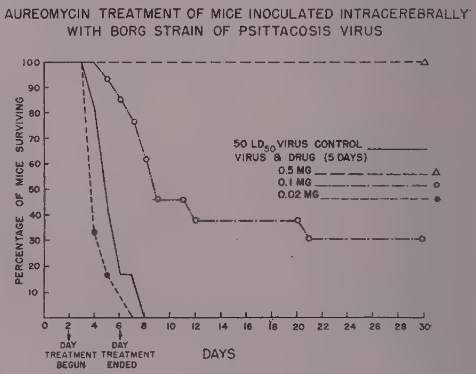

FIG. 1.

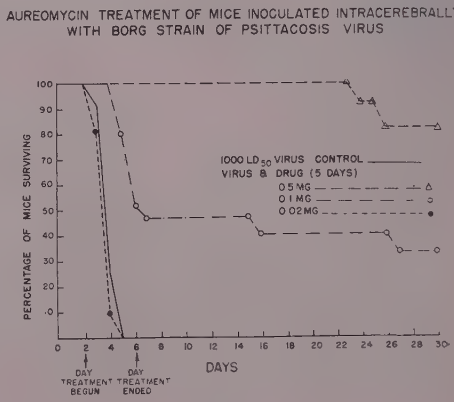

FIG. 2.

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† The results of more detailed studies on the tissue distribution and excretion of aureomycin in animals receiving repeated doses will be reported by Dr. J. Forgacs, Camp Detrick, Md. in a later publication.

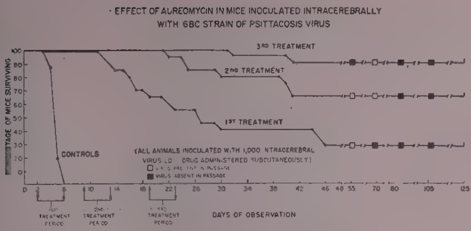

FIG. 3.

Discussion. The data indicate that aureomycin had a definite therapeutic effect in both eggs and mice infected with several virus strains of the psittacosis-LGV group. The results obtained in the egg studies seem particularly significant since large numbers of treated embryos survived until time of hatching while a majority of untreated controls died. These observations are in contrast to those previously reported for the psittacosis-LGV viruses tested against penicillin and sulfadiazine(2), where the treated embryos showed significant delay in death but seldom survived the prehatching observation period.

All the virus strains injected in high dilution were nearly completely inactivated by aureomycin. As the virus concentration increased, this inactivation was manifested only by the survival of drug treated embryos but not by the titer of the virus in them. These data suggest a quantitative relationship between the concentration of virus and the dosage of aureomycin used. With strains 6BC, Borg, Gleason, S-F, P-207 and Cal 10, virus was present in high titer in surviving drug treated embryos, while strains LGV, 12xN, Baker and Nigg yielded titers so low as to suggest almost complete inactivation by the drug in dilutions tested. These results are understandable since, in normal titrations of the strains, the former group yield titers far in excess of those observed for the latter.

The results of studies in mice were in agreement with those obtained in chick embryos. Treatment of infected animals with 0.5 mg doses of drug not only extended the A.D.D., but markedly reduced the percentage of deaths. Gradations in these therapeutic effects were demonstrated by using lesser amounts of drug. It is of particular interest that the drug was not detectable in brain tissues even though beneficial effects were apparent after intracerebral virus inoculation. This was true even after five successive daily injections of drug. Thus, it seems that drug concentrations of less than 0.3 μg per ml were sufficient to prevent death, but not to sterilize brain tissue although it is possible that aureomycin was present in higher concentrations than indicated, but masked by some substance present in brain tissue.

Summary. 1. Aureomycin was effective, in both embryonated eggs and in mice, in controlling experimental infections with 10 strains of virus in the psittacosis-lymphogranuloma venereum group. 2. Drug activity was evidenced in both the comparative titers of treated and untreated embryos, and in the titer of the virus recovered from these treated embryos. 3. In mice, multiple doses of 0.5 mg of drug lengthened significantly the life span of treated groups of animals (Borg, S-F, Gleason, 6BC, P-207), as compared to untreated controls. Gradations in these therapeutic effects were demonstrated by lesser amounts of drug. 4. The survival of treated mice as compared to untreated controls was markedly increased by repeated cycles of drug treatment.

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Effects of Some Substituted Thioureas on Alpha-naphthylthiourea (ANTU) Toxicity to Rats. (18394)

JACK P. SAUNDERS AND RAYMOND C. SPAULDING.[*]
(Introduced by William H. Chambers.)
(With the technical assistance of Frank Tittle.)
From the Toxicology Section, Medical Division, Army Chemical Center, Md.

It has been reported from this laboratory that relatively nontoxic thioureas related in structure to ANTU are effective in reducing mortality in rats when given before, simultaneously with or after toxic doses of the rodenticide(1). Two alkyl singly N-substituted thiourea derivatives, allyl thiourea and isopropyl thiourea, were most effective in reducing mortality in rats when given simultaneously with or one hour after the injection of two LD_{50}'s (5 mg/kg) of ANTU. It was suggested that these compounds confer their protection against ANTU poisoning by virtue of their similar structure and act as competitive antagonists against the rodenticide.

We have recently obtained three additional substituted thioureas, ethyl thiourea, n-butyl thiourea and 1-ethyl-1-phenyl thiourea, and have investigated the effects of these compounds on ANTU toxicity to rats.

Materials and methods. The compounds[†] used in this study included 2 singly substituted thioureas (ethyl thiourea and n-butyl thiourea) and one 1,1 disubstituted thiourea (1-ethyl-1-phenyl thiourea). Wistar, male, albino rats, weighing from 200 to 300 g, were used throughout these experiments. The animals were born and raised in our animal colony and maintained for no less than one month on a diet previously described(2). Injections of ANTU and the compounds tested were given by the intraperitoneal route with constant nontoxic quantities of propylene glycol (1.0 ml/kg) as the vehicle. Simultaneous injections of ANTU and test-compounds were made separately in different areas of the abdomen. Tests were conducted in groups of 8 animals. Control animals were injected on each test-day with the same dose of ANTU (2 LD_{50}'s[‡] = 5.0 mg/kg) as given to rats subjected to treatment. One set of controls served for each compound tested. Both control and experimental (treated) groups were observed for a period of 5 days following the injection of ANTU.

Results and discussion. Of the 3 compounds tested, 1-ethyl-1-phenyl thiourea (EPTU) was most effective as an antidote when given as much as 2 hours after ANTU (Table I). No animal treated with this compound died. Both ethyl thiourea and n-butyl thiourea showed significant reductions in mortality when administered in doses of 25 mg/kg and 50 mg/kg, but when compared with EPTU at the higher dose, little protection was given when administered 2 hours after ANTU, as shown in Table II. This may have been due to increased toxicity at the higher dose level.

Rats that had been given ANTU followed by EPTU had an amazing recovery. A rat poisoned with ANTU usually shows signs of uneasiness in an hour and, at the end of 2 hours, is quite dyspneic. Death usually occurs within 12 hours. This course of events was followed partly by the rats given EPTU 2 hours after ANTU. This is to say that prior to the EPTU injection the rats were dyspneic and almost completely inactive. Within one hour after EPTU injection normal respiration was restored and by the end

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[*] Present address: Department of Surgery, University Hospital, Baltimore, Md.

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[†] Ethyl thiourea, n-butyl thiourea and 1-ethyl-1-phenyl thiourea were supplied through the generosity of Sharples Chemicals, Inc., Philadelphia, Pa. E. I. du Pont de Nemours and Co., Wilmington, Del., kindly furnished the ANTU.

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[‡] LD_{50} = 2.47 mg/kg, calculated by the method of Bliss(3).

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TABLE I. Effect of Substituted Thioureas When Given to Rats Simultaneously With, One or Two Hours After ANTU.*

Compound	Dose† mg/kg*	When given	Mortality Control	Mortality Experimental	P‡
Ethyl thiourea	25	Simultaneously	7/8	1/8	$<.02$
,,	25	1 hr after ANTU		3/8	$>.10$
,,	25	2 hr after ANTU		1/8	$<.02$
,,	50	Simultaneously	7/8	1/8	$<.02$
,,	50	1 hr after ANTU		2/8	$<.05$
,,	50	2 hr after ANTU		6/8	$>.10$
n-butyl-thiourea	25	Simultaneously	7/8	0/8	$<.01$
,,	25	1 hr after ANTU		1/8	$<.02$
,,	25	2 hr after ANTU		3/8	$>.10$
,,	50	Simultaneously	7/8	2/8	$<.05$
,,	50	1 hr after ANTU		0/8	$<.01$
,,	50	2 hr after ANTU		4/8	>10
l-ethyl-l-phenyl thiourea	50	Simultaneously	7/8	0/4	$<.01$
,,	50	1 hr after ANTU	14/16	0/16	$<.01$
,,	50	2 hr after ANTU		0/16	$<.01$

* Inj. intraper. 5.0 mg/kg equivalent to an expected mortality of ca 90%.

† Inj. intraper. in a constant non-toxic quantity of propylene glycol (1.0 ml/kg).

‡ P $<.05$ indicates significant difference between experimental and control groups.

of 2 hours the animals were moving around the cage. The next morning they could not be distinguished from normal animals. Rats given EPTU simultaneously with the rodenticide developed no symptoms of ANTU poisoning.

Of all the compounds studied in this laboratory(2,4), EPTU appears to be the most effective antidote against ANTU poisoning in rats. Although EPTU has an LD_{50} approximately 1/100 that of ANTU,§ the structures of the two compounds are quite similar. We have reported previously that lethal quantities of certain thiourea derivatives having structures similar to that of ANTU produce pulmonary edema and pleural effusion, which are the main symptoms of ANTU poisoning. The compounds studied here are not exceptions, and it seems probable that thiourea derivatives confer antidotal protection by acting as competitive antagonists against ANTU. Because of the effectiveness of EPTU as an antidote against ANTU poisoning in rats, it was hoped that EPTU could be used in cases of ANTU poisonings of domestic animals. However, preliminary experiments in this laboratory with a limited number of dogs have shown EPTU to be ineffective.

Summary. 1. l-ethyl-l-phenyl thiourea (EPTU) administered in an intraperitoneal dose of 50 mg/kg was effective in significantly reducing mortality and toxic symptoms in rats when given simultaneously with, one hour or 2 hours after two LD_{50}'s (5 mg/kg) of ANTU. All animals receiving EPTU survived. 2. Ethyl thiourea and n-butyl thiourea also conferred significant but less uniform protection when given in doses of 25 and 50 mg/kg.

TABLE II. Comparison of Protective Action of 50 mg Doses of Ethyl Thiourea (I), n-butyl Thiourea (II), and l-ethyl-l-phenyl Thiourea (III). (P = Probability by chi-square test(5). P $<.05$ indicates significant difference between 2 drugs).

Drug	Treatment	Chi square (X^2)	P
I vs II	Simultaneous	.33	$>.50$
I vs III	,,	.08	$>.70$
II vs III	,,	.50	$>.30$
I vs II	1 hr after ANTU	1.641	$>.20$
I vs III	,, ,, ,,	3.857	$<.05$
II vs III	,, ,, ,,	.13	$>.70$
I vs II	2 hr after ANTU	.27	$>.50$
I vs III	,, ,, ,,	7.87	$<.01$
II vs III	,, ,, ,,	9.13	$<.01$

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§ LD_{50} = 297 ± 63 mg/kg, calculated by the method of Bliss.

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Treatment of Pernicious Anemia with Citrovorum Factor.* (18395)

Leo M. Meyer, Conrad M. Brahin, and Arthur Sawitsky.

From the New York University Medical Service, Goldwater Memorial Hospital, Welfare Island, New York.

Sauberlich and Bauman(1) first described a factor (CF) present in liver which is necessary for the growth of *Leuconostoc citrovorum* 8081. The relationship of folic acid and ascorbic acid to this factor has been recently reported by Sauberlich(2), and Nichol and Welch(3,4).

The present study was undertaken to investigate the effect of parenterally administered CF to 5 patients with pernicious anemia in relapse. Each case showed hyperchromic macrocytic anemia, histamine-fast achlorhydria, and megaloblastic bone marrow. Roentgen examinations of the gastrointestinal tract, urinalyses and blood chemistry determinations disclosed no abnormalities. The concentrate contained 20 million CF units per ml.† Four of the patients received 1 ml of the concentrate intramuscularly, daily, and the fifth was given one-half of this dose. Hemoglobin and red blood cell determinations were made three times a week, leukocyte counts once a week, and reticulocyte estimations daily.

Procedure. A. R., white female, age 62, was admitted to the hospital with a history of Paget's disease of 30 years' duration. The patient had no symptoms referable to the anemia. Physical examination revealed the typical findings of Paget's disease and an atrophic smooth tongue. Neurologic status was normal insofar as it could be determined. The initial blood examination showed hemoglobin 4.5 g per 100 ml and red blood cells 1.44 million per cmm. She received 1 ml of CF intramuscularly, daily. Her appetite was increased on the fourth day. She became more cheerful and talkative than she had been in many years. Her strength gradually improved and there was an increased sense of well being. The reticulocyte response was submaximal (15.3%). The hemoglobin and red blood cells rose slowly, and remained stationary at a level of 8.5 g and 2.50 million per cmm, respectively, after 42 days of treatment (Fig. 1). Accordingly, the dose of medication was doubled and a secondary rise in hemoglobin and erythrocytes ensued. During the first phase of treatment with 1 ml of CF daily the leukocytes remained below 5,000 per cmm. After the dose of CF was increased to 2 ml per day the white blood cells rose to a level between 5,000 and 6,000 per cmm. After 2 months of treatment the hematologic values did not reach normal values. The neurologic status was unchanged. Clinically the patient was improved.

E. Mc., white female, age 63, was a known case of pernicious anemia since 1936 who had intermittent and frequently incomplete treatment because of her failure to attend the clinic regularly. She received no therapy for 4 months prior to the present admission. Her chief complaints were weakness and a burning sensation in both legs. Physical examination revealed an enlarged liver 4 cm below the right costal border, loss of position and vibratory senses in both lower extremities, weakness and flexion deformities of both legs, and weakness of ankle jerks bilaterally. The initial hemoglobin and red blood cell values were 9.0 g per 100 ml and 2.60 million per cmm, respectively. She was given 1 ml of CF intramuscularly, daily. Improvement in appetite and sense of well being was not ob-

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* Supplied by Dr. J. M. Ruegsegger, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

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† One CF unit is equivalent to 0.00015 μg of the anhydrous free acid of CF(5).

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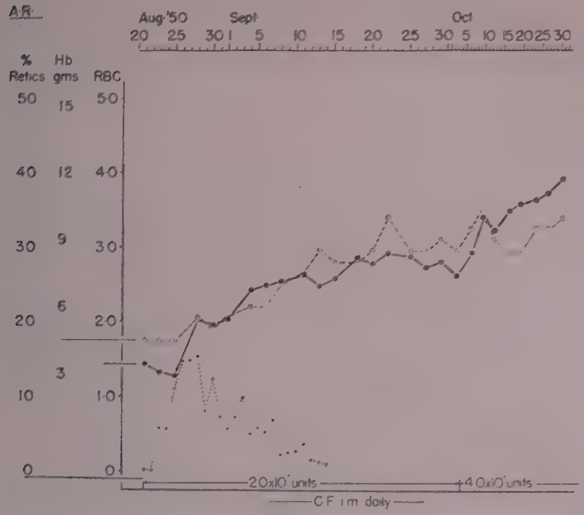

Fig. 1.

Hematologic data on patient A.R. with pernicious anemia treated with 20 and 40 million units of CF intramuscularly daily. The reticulocyte response was submaximal and the hematologic values were suboptimal.

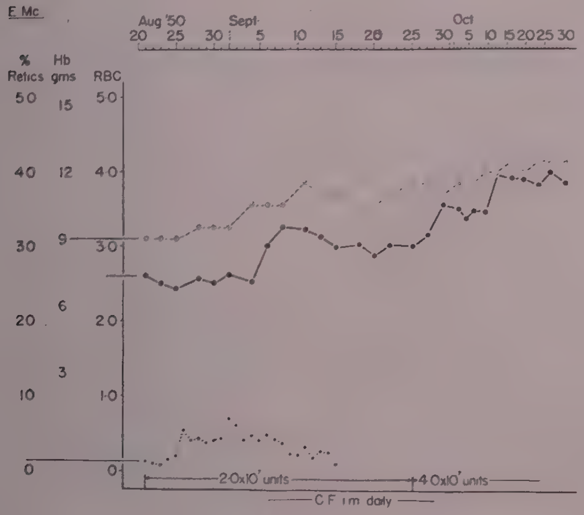

Fig. 2.

Hematologic data on patient E.Mc. with pernicious anemia treated with 20 and 40 million units of CF intramuscularly daily. The reticulocyte response was submaximal and the hematologic values were suboptimal.

served until the tenth day of treatment and was very slow thereafter. A prolonged submaximal reticulocytosis (up to 7%) was noted (Fig. 2). The hemoglobin and erythrocyte values rose slowly to a stationary maximal level of 11.5 g per 100 ml and 3.05 million per cmm, respectively, after 35 days of treatment. The neurologic status improved in that the patient no longer complained of burning of her legs. The dose of CF was increased

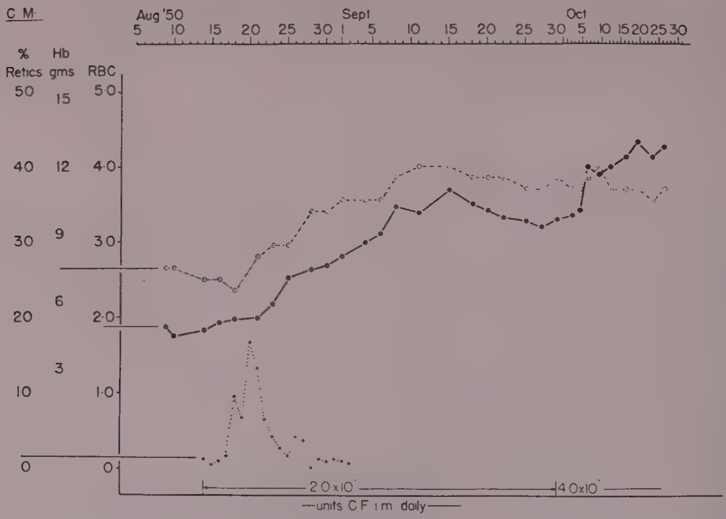

FIG. 3.

Hematologic data on patient C.M. with pernicious anemia treated with 20 and 40 million units of CF intramuscularly daily. The reticulocyte response was submaximal and the hematologic values were suboptimal.

to 2 ml, intramuscularly, daily. A rise in the amount of hemoglobin and the number of red blood cells followed; at the end of 66 days of treatment they were 12.5 g per 100 ml and 4.00 million per cmm, respectively.

C. M., white female, age 60, was admitted to the hospital because of Parkinson's disease, cerebral arteriosclerosis, and a history of left hemiplegia 6 years previously. Physical examination disclosed generalized rigidity and tremors. Numerous purpuric areas were seen. Neurologic examination showed a positive Babinski sign on the left side, diminution of position sense in toes, and vibratory sense reduced in both legs. The initial blood examination showed hemoglobin 7.5 g per 100 ml and erythrocytes 1.86 million per cmm, respectively. The patient was given 1 ml of CF, intramuscularly, daily. Within 3 days she felt stronger. No new purpuric spots appeared. Two days later her appetite was increased. After this the patient showed rapid clinical improvement and gained weight. Six weeks later the Babinski sign was negative bilaterally. The patient was able to detect vibration in both legs and position sense was now intact in both feet. The reticulocyte response was submaximal (16.6%). The hemoglobin and erythrocyte values rose to a stationary level of 11.5 g and 3.30 million per cmm, respectively, after 56 days (Fig. 3). The dose of CF was increased to 2 ml daily. A rise of red blood cells to 4.20 million per cmm followed but the hemoglobin was unchanged after a total course of treatment of 84 days. The neurologic status showed no further improvement.

O. J., white male, age 70, was admitted to the hospital completely disoriented and with no available history. On examination he was pale and weak. Neurologic examination was unreliable. The initial hemoglobin and erythrocyte values were 5.0 g per 100 ml and 1.75 million per cmm, respectively. He was given 500 ml of blood and then treated with 1 ml of CF, intramuscularly, daily. Clinical improvement was observed on the third day as evidenced by an increase in appetite, strength, and sense of well being. He was out of bed on the sixth day. A maximal reticulocyte count was observed (26.7%). The hemoglobin level and red blood cell count rose to 12.5 g per 100 ml and 4.60 million per cmm, respectively, after 47 days of treatment (Fig. 4). The patient died suddenly at home of a coronary thrombosis.

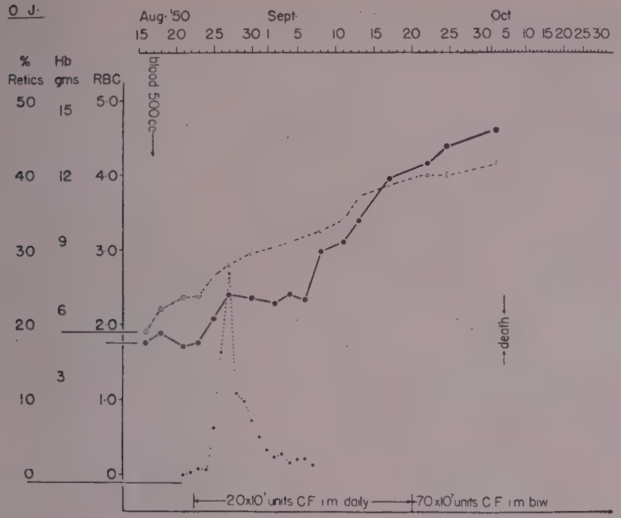

FIG. 4.

Hematologic data on patient O.J. with pernicious anemia treated with 20 million units of CF intramuscularly daily. The reticulocyte response was maximal and the hematologic values reached near normal levels after 47 days of treatment.

B. H., white female, age 63, was admitted to the hospital because of anorexia, weakness, loss of 50 lb, and numbness and tingling of fingers and toes. Physical examination revealed mild Parkinsonism, smooth tongue, and absence of position and vibratory senses in toes. The initial hemoglobin and erythrocyte values were 4.0 g per 100 ml and 1.01 million per cmm, respectively. The patient was transfused with 500 ml of blood and treated with 0.5 ml of CF, intramuscularly, daily. Subjective improvement was reported on the eighth day, at which time a submaximal reticulocyte peak (13.7%) was noted (Fig. 5). The following day she suddenly became dehydrated. She was transfused with 500 ml of blood and the dose of CF was increased to 1 ml per day. A secondary reticulocytosis (13.0%) followed. At this time the urine contained large numbers of clumps of leukocytes. She was treated with penicillin, streptomycin, fluids, and large doses of vitamin B_{12}. A third reticulocytosis (14%) occurred 2 days later. She became comatose and died 3 days later.

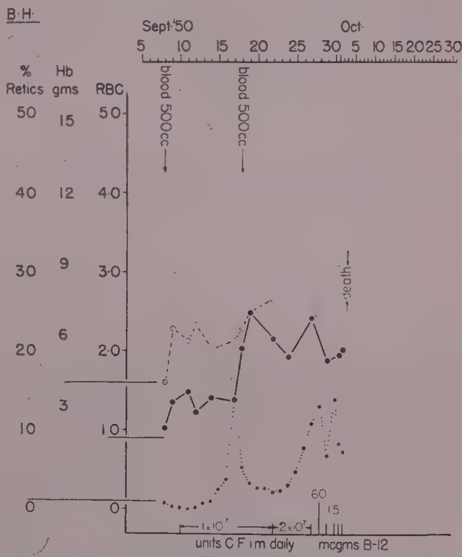

FIG. 5.

Hematologic data on patient B.H. with pernicious anemia treated with 10 million units of CF intramuscularly daily. The reticulocyte response was poor. The daily administration of 20 million units of CF produced a second reticulocyte response. The further administration of large doses of vitamin B_{12} was followed by a third reticulocyte peak.

Discussion. From the foregoing data it is evident that the daily administration of 10 or 20 million units of citrovorum factor intramuscularly was insufficient to produce a satisfactory reticulocytosis in 4 of 5 patients with pernicious anemia in relapse. Three of these persons reached stationary blood levels below 12.0 g of hemoglobin per 100 ml and 4.0 million erythrocytes per cmm, respectively, after 35, 42, and 66 days, respectively. A fourth patient appeared to have attained near normal values when he died suddenly of a vascular accident. The fifth patient clearly showed a poor response to daily administration of 10 million units of citrovorum factor. In view of the intercurrent infection the efficacy of the 2 ml dose cannot be evaluated in this subject. Because of complicating associated disease clinical and subjective reactions were difficult to estimate. Improvement in appetite, strength, and well being appeared to follow in all instances but not so dramatically as with adequate amounts of liver extract, vitamin B_{12}, or folic acid. This is probably due to the inadequacy of 20 million units per day as an optimal dose. Significant increases in erythrocytes were observed in 3 patients when the citrovorum factor was increased to 40 million units a day. Further evidence of the inadequacy of the initial dose was shown in patient A. R. in whom leukopenia persisted until the medication was increased to 2 ml a day. Neurologic examinations were difficult to evaluate because of associated complicating diseases. In one case, however, a distinct improvement followed treatment with 20 million units a day.

Summary. The daily administration of 10 or 20 million units of citrovorum factor to 5 patients with pernicious anemia in relapse usually is followed by a submaximal reticulocyte response, suboptimal levels of hemoglobin and red blood cells, and moderate to good clinical remissions. In one case a theoretically maximal reticulocyte response followed the daily administration of 20 million units of citrovorum factor. Neurologic signs improved in one patient. Although the daily administration of 40 million units produced a further rise in erythrocytes in 3 cases, normal blood values were not attained.

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Effect of Photodynamic Action on the Viscosity of Desoxyribonucleic Acid.* (18396)

HENRY KOFFLER AND IRMA L. MARKERT.

From the Department of Biological Sciences, Purdue University, Lafayette, Ind.

Recent observations(1) have indicated the possibility that photodynamic action (the combined effect of radiations and sensitizing agents) is mutagenic as well as lethal to certain bacteria. Since genes are thought to contain desoxyribonucleic acid (DNA), it is conceivable that mutations occur when the native structure of the DNA is altered. Some strength to this contention is given by the reports that several mutagenic agents, such as ultraviolet radiations(2), X-rays(3,4), mustard gas and the nitrogen mustards(5,6), and peroxides(7) apparently are able to

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* Supported in part by a grant from the Tippecanoe County Cancer Society made through the Lions Club of Lafayette.

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depolymerize highly viscous solutions of DNA *in vitro*. Even more dramatic were the observations made by Errera(8) and Limperos and Mosher(9). The former demonstrated that nuclei of chicken erythrocytes became less rigid on exposure to X-rays, and the latter that thymus DNA isolated from rats 24 hours after they had been irradiated with X-rays was almost completely depolymerized. The study reported here led to the conclusion that also photodynamic action can reduce the viscosity of DNA solutions.

Experimental. The preparation of the highly polymerized DNA used was purchased from the Worthington Biochemical Laboratory, where it was isolated essentially by the method of Mirsky and Pollister(10). The photodynamically active compounds tested were the dyes eosin (E) and methylene blue (MB), the slightly carcinogenic hydrocarbon 1,2-benzanthracene (BA), and the highly potent carcinogen 20-methylcholanthrene (MC). All substances were used at a concentration of 1:100,000, the dyes dissolved in water, and the hydrocarbons as colloidal suspensions prepared from 1:1000 acetone solutions. To determine the effect of photodynamic action DNA was irradiated in the presence of one of the photodynamically active compounds tested. Three types of control were used. One determined the effect of radiation alone, the second the effect of the compound alone, and the third the fate of the DNA in the absence of either radiation or sensitizer but otherwise under the same conditions as the exposed DNA. Test tubes containing the desired combinations were placed on a Burrell Wrist Action Shaker, and agitated to allow uniform exposure of the DNA. A source of radiation was placed exactly 10 cm from the center of the tubes. For E and MB this was a 100 watt light bulb; for the hydrocarbons a Hanovia quartz lamp screened with a Corning glass filter to transmit radiations near 3600 Å. In one experiment, designed to study the effect of BA and ultraviolet radiation, the samples were placed in quartz tubes and exposed to radiations from the unscreened quartz lamp at a distance of 25 cm from the center of the tubes. Heat produced by these sources was dissipated by a fan placed behind a radiator through which cold water circulated. During irradiation samples were removed at intervals, and their relative viscosity was determined with an Ostwald viscosimeter. The suspensions were allowed to equilibrate at 25°C for 5 minutes. An average of 3 readings was used in the calculations.

Results. Fig. 1 illustrates that BA in conjunction with "white" light causes a decrease in the relative viscosity of DNA. The behavior of the controls indicated that neither BA nor light alone had any depolymerizing effects during the experimental period. DNA in the absence of the chemical and protected from radiations by a cover (but otherwise treated exactly like the exposed DNA) also

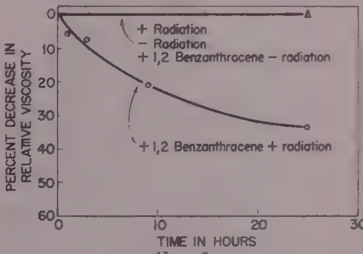

Fig. 1.

Effect of 1,2-benzanthracene and "white" light on the relative viscosity of desoxyribonucleic acid.

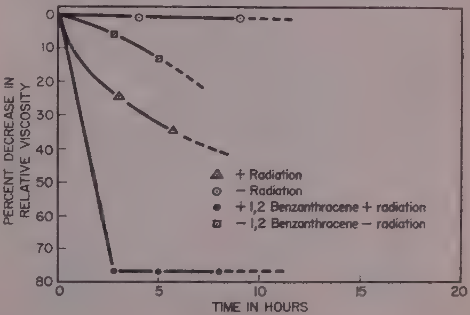

Fig. 2.

Effect of 1,2-benzanthracene and ultraviolet radiation on the relative viscosity of desoxyribonucleic acid.

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TABLE I. Effect of Photodynamic Action on the Viscosity of DNA.

Sensitizer*	Radiation*	Time to give 20% decrease in rel. viscosity, hr
Eosin	"White" light	7.3
Methylene blue	" "	55
1,2-benzanthracene	ca. 3600 Å	7.6
1,2-benzanthracene	ca. 2537 Å	0.7
None	ca. 2537 Å	2.4
20-methylcholanthrene	ca. 3600 Å	48

* In the time necessary to give a 20% decrease in relative viscosity by the sensitizer plus radiation the following decreases in relative viscosity (in %) were obtained in the various controls: no treatment, 0-1.7; "white" light, 0-3.5; 3600 Å, 0; eosin, 7.4; methylene blue, 5.8; 1,2-benzanthracene, 0; or 20-methylcholanthrene, 0.

remained unaffected. In the case of E and MB the controls showed a small decrease in viscosity, and this was most marked for the shielded samples that were shaken in the presence of the dyes. Some depolymerization may have occurred while the viscosity measurements were being made in the light.

Fig. 2 shows a similar experiment with BA and ultraviolet radiation (essentially at 2537 Å). Ultraviolet radiation alone reduces the viscosity of DNA suspensions, but BA accentuates this effect very strikingly. Table I summarizes data that were taken from such graphs as shown in Fig. 1 and Fig. 2. No attempt was made in this study to find the optimum conditions for depolymerization. Such important factors as the affinity of the sensitizing substances for DNA, the wavelength of radiation used, etc. were not fully considered. The information in Table I, therefore, should be considered more as qualitative than as quantitative.

Comments. The observation that photodynamic action *in vitro* decreases the viscosity of DNA (which may mean that DNA becomes depolymerized) should be interpreted with caution. Although it is possible that such an alteration of the DNA present in genes is the cause of lethal and other mutations, such an explanation still awaits confirmation from experiments performed on living cells. It is known that carcinogenic hydrocarbons tend to be photodynamically active(11-17). This activity has been said(12) to depend on light-produced, water-soluble products of these hydrocarbons, and the possibility should be considered that such impurities are responsible also for the effects observed in this investigation. It would of course be premature to speculate on a possible connection between the carcinogenicity of certain hydrocarbons and their ability in the presence of radiation to decrease the viscosity of DNA suspensions.

Summary. Suspensions of desoxyribonucleic acid become less viscous when they are irradiated in the presence of photodynamically active compounds, such as eosin, methylene blue, 1,2-benzanthracene, or 20-methylcholanthrene. This apparent depolymerization may be involved in the lethal and possible mutagenic effects of photodynamic action.

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Fungicidin, an Antibiotic Produced by a Soil Actinomycete. (18397)

Elizabeth L. Hazen and Rachel Brown.
From the N. Y. Branch Laboratory, and the Division of Laboratories and Research, New York State Department of Health, Albany.

In a search among freshly isolated actinomycetes for antagonists to pathogenic fungi (1-3), an antibiotic substance, designated *fungicidin,* has been obtained from a strain (No. 48240) isolated from a farm soil in Fauquier County, Va.(4). Fungicidin is both fungistatic and fungicidal; and apparently lacks antibacterial activity.

Actinomycete No. 48240, belongs to the genus *Streptomyces*(5); the species has not been determined. Oval spores are produced in chains from curved and spiral-forming hyphae. (Fig. 1). Grown on Sabouraud's glucose agar at ±28°C for 7 days, the colony is heaped and folded and the aerial mycelium is white, but later at sporulation it is gray; on the reverse, the color at first is light tan, but after 2 weeks it is dark brown and the medium is darkened throughout. There is no hemolysis on blood agar medium. Fig. 2 shows zones of inhibition produced by this streptomyces against the 2 assay species *Cryptococcus neoformans* and *Candida albicans.* When grown in a glucose-tryptone broth(3) in shallow layers,* the stationary culture yields two antibiotics both active against fungi. From the liquors one substance resembling actidione(2,6) has been obtained. From the surface growth a second substance with much broader antifungal activity has been extracted; this is designated fungicidin.

Isolation. The surface growth from 5- to 7-day-old broth cultures is harvested, heated at 70°-72°C for 10 minutes, pressed between filter papers to remove adhering medium, and extracted several times with methanol. The antibiotic in the pooled extracts is purified by a process that involves repeated fractional precipitations with ethyl acetate, washing of the precipitates with 0.85% NaCl solution, dissolution in methanol, and fractional precipitation with ether. All procedures are carried out in the cold so far as practicable.

Chemical and physical properties. Crude fungicidin is obtained in a yield of 30 to 50 mg per liter of culture medium. It is a fine yellow powder that can be stored in the cold for several months without loss of activity. It is only sparingly soluble in methanol and ethanol, and relatively insoluble in most organic solvents and water. Very

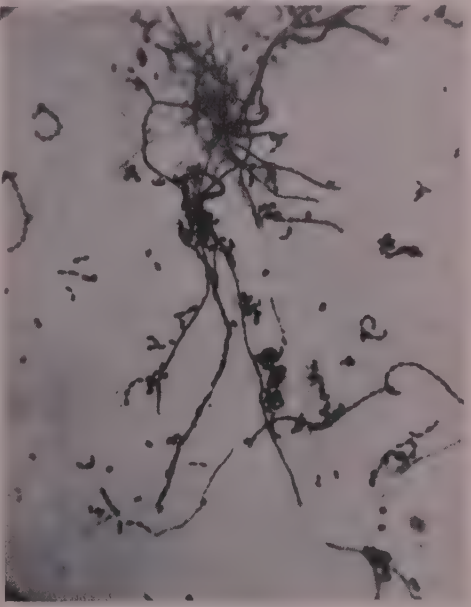

Fig. 1.
Streptomyces sp. No. 48240 grown on Sabouraud's glucose agar.

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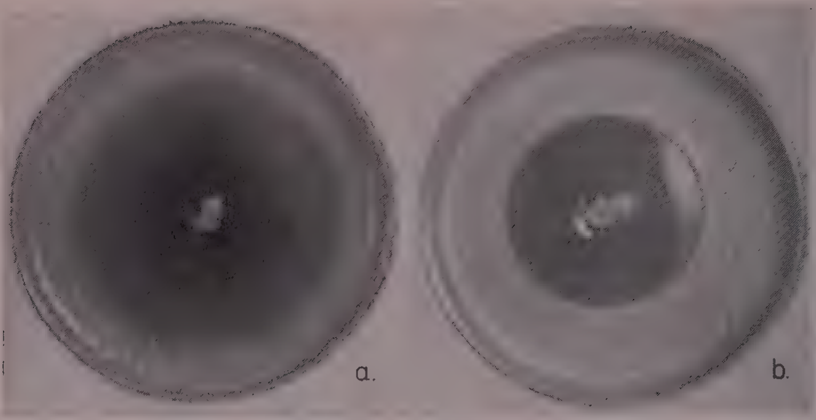

FIG. 2. Zones of inhibition of *Streptomyces* sp. No. 48240 against:
a. *C. neoformans*. b. *C. albicans*.

unstable aqueous solutions, however, can be prepared at pH 2 or pH 9. In the present crude state, preparations of fungicidin contain about 1.5% of Kjeldahl nitrogen. Sulfur and the halogens have not been detected. Fungicidin shows strong reducing properties. It fails to react in tests for carbohydrate or protein. The molecule is relatively small since it may be ultrafiltered through fine gradocol membranes.

In vitro activity. Materials and methods. A stock suspension of fungicidin containing 5 mg/ml is prepared by dissolving the powder in N/50 HCl and adjusting to pH 7.2 with 0.1% Na_2CO_3. This is kept in a refrigerator and used for only 72 hours. Dilutions are prepared in sterile distilled water. The sensitivity of *C. neoformans* and *C. albicans* to fungicidin was determined in broth cultures. Serial 2-fold dilutions were made in 4.5 ml glucose-tryptone broth and heated for 10 minutes at 70°C for sterilization. To 2 series of tubes was added, respectively, 0.5 ml of a saline suspension of *C. neoformans* and *C. albicans* in concentrations of 1,000,000 and 300,000 cells per ml. Readings of visible growth were made after 5, 24, 72, and 96 hours' incubation at ±28°C. The sensitivity (7) was recorded as the least amount of fungicidin inhibiting growth, as evidenced by absence of gross turbidity after 96 hours.

The antimicrobial activity of fungicidin was determined by the agar dilution method of Reilly, Schatz, and Waksman(8) except that infusion agar was employed for the bacterial cultures and the temperature of incubation was 35°-36°C. The effect of fungicidin upon the growth of *C. neoformans* was determined in broth cultures. Two sets of serial 2-fold dilutions in glucose-tryptone broth were inoculated, respectively, with 10,000,000 and 1,000,000 microorganisms per ml. After incubation for 3, 4, 5, 24, and 48 hours, aliquot portions were removed and plated on glucose-tryptone agar. Colony counts were made after 48 hours of incubation. The effect of defibrinated horse blood and serum was determined as follows: 0.1 ml of fungicidin diluted in sterile saline was added to a series of tubes containing 0.25 ml of whole blood or serum and to each dilution was added 0.05 ml of a 48-hour glucose-tryptone broth culture of *C. neoformans* containing 26,000,000 microorganisms/ml. The tests were incubated for 48 hours with frequent shaking, after which the contents were streaked on plates of Sabouraud's glucose agar which were incubated for 48 hours.

Results. The data presented in Tables I and II indicate that fungicidin is strongly fungistatic against a wide variety of saprophytic and pathogenic forms. Amounts such

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7. Lenert, T. F., and Hobby, G. L., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 235.

8. Reilly, H. C., Schatz, Albert, and Waksman, S. A., *J. Bact.*, 1945, v49, 585.

TABLE I. Fungistatic Action of Fungicidin.

Test species	Sensitivity: μg of fungicidin per ml at ±28°C 72 hr	96 hr
C. neoformans	1.56	1.56
C. albicans	3.12	3.12

TABLE II. Antifungal Spectrum of Fungicidin.

Fungi	Strain No.	Least amt inhibiting growth, μg/ml
Cryptococcus castellanii	45232	3.13
Cryptococcus glutinis	4676	1.56
Candida guilliermondi	45211	3.13
Candida krusei	45214	6.25
Candida stellatoidea	45213	3.13
Saccharomyces cerevisiae	45217	3.13
Sporobolomyces salmonicolor	4550	3.13
Schizosaccharomyces octosporus	45231	1.56
Endomycopsis fibuliger	45230	3.13
Geotrichum lactis	47462	6.25
Aspergillus fumigatus	50248	6.25
Penicillium notatum	43281	3.13
Penicillium sp.	50526	13
Penicillium claviforme	49470	3.13
Rhizopus nigricans	50524	3.13
Fusarium sp.	50527	3.13
Alternaria sp.	50523	1.56
Cephalosporium sp.	45226	25
Phoma sp.	50522	6.25
Ceratostomella ulmi (plant pathogen)	50525	6.25
Hormodendrum sp.	4893	3.13
Histoplasma capsulatum (yeastlike)	4894	1.56-
Blastomyces dermatitidis (yeastlike)	45223	1.56
Paracoccidioides brasiliensis (yeastlike)	45224	1.56
Coccidioides immitis (spherules)	50521	6.25
Cryptococcus neoformans	45215	1.56
Candida albicans	4657	3.13
Trichophyton mentagrophytes	45141	6.25
Trichophyton rubrum	4516	6.25
Trichophyton rosaceum	4974	3.13
Epidermophyton floccosum	44253	1.56
Microsporum audouini	4896	3.13
Microsporum canis	4817	13
Sporotrichum schenckii (mycelial) (yeastlike)	50251	13
Monosporium apiospermum	45221	100
Allescheria boydii	48102	>100
Phialophora verrucosa	45229	13

as 1.56 to 6.25 μg/ml were effective against many fungi. Inhibition of growth of the following bacteria, however, was not obtained even with 100 μg/ml: *Staphylococcus aureus* (No. 45142), *Streptococcus hemolyticus* (No. 44131), *Bacillus subtilis* (No. 45137), *Bacillus cereus* var. *mycoides* (No. 48294), *Salmon-*

TABLE III. Therapeutic Activity of Fungicidin in Mice Infected with *C. neoformans*.

Infection		Therapy				% survival weeks							Findings at autopsy		
													Microscopic and cultural		
Date	No.* of microorganisms	Dates	No. of inj.	Total amt. inj. mg/kg	No. of mice, 20 g	1	2	3	4	5	6	7	Brain	Blood	Gross lesions Liver, spleen, kidney
10/16/50	800,000	10/16/50 through 12/4/50	28†	985	10	100	100	100	100	100	90	90	+ (1/1)	— (1/1)	None (1/1)
10/16/50	800,000	10/17/50 through 12/4/50	30‡	1135	10	100	100	80	70	40	20	10	+ (9/9)	— (9/9)	None (9/9)
10/16/50	800,000		0	0	10	100	90	10	0	0	0	0	+ (10/10)	+ (10/10)	+++ (10/10)
	0	10/16/50 through 11/13/50	25	735	5	100	100	100	100	100	100	100			

* Determined by hemocytometer.

† Mice received 13 inj. the first week, 5 the second, 4 the third, 2 the fourth, and 4 during the next three weeks.

‡ Mice received 11 inj. the first week, 7 the second, 5 the third, 3 the fourth, and 4 during the next 3 weeks.

Infection by intravenous route. Therapy by subcutaneous route. Doses ranged from 250-2000 μg per mouse.

+ Microorganisms typical in morphology. — No microorganisms. +++ Many small white nodular masses on surface of organs.

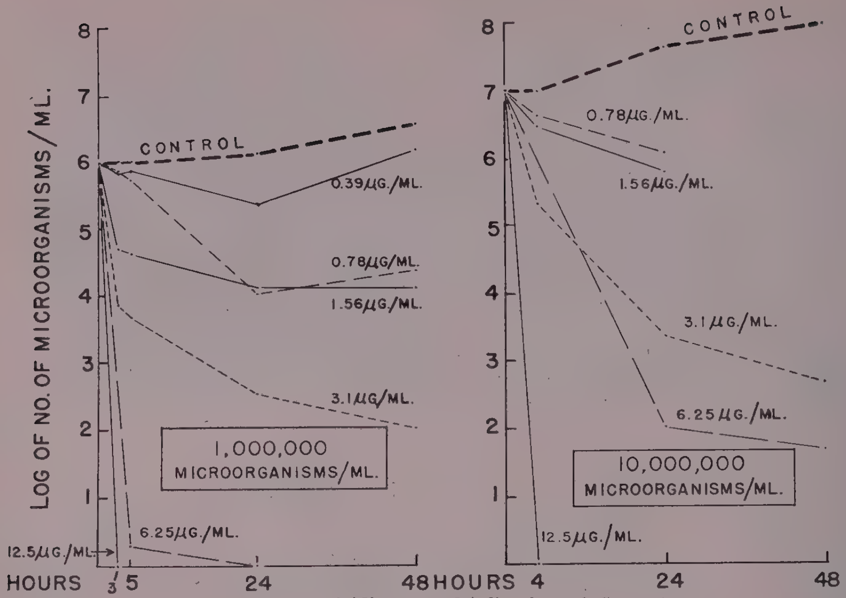

FIG. 3. Effect of fungicidin on multiplication of *C. neoformans.*

ella typhosa (No. 38351), *Shigella paradysenteriae* (No. 45300), *Bacterium mucosum capsulatum* (No. 4767), and *Bacillus circulans* (No. 48205). Growth of a strain of *Mycobacterium tuberculosis* (No. 50539) freshly isolated from a human lung was not inhibited in Dubos medium containing 100 µg of fungicidin/ml.

The growth curves in Fig. 3 indicate fungicidin to be strongly fungistatic and fungicidal. In the presence of 1,000,000 microorganisms, 6.25 µg brought about a progressive decline in the population, with sterilization in 24 hours; and, with a ten-fold increase in microorganisms, 12.5 µg produced sterilization within 4 hours. No evidence was obtained that incubation of fungicidin in the presence of defibrinated horse blood or serum modifies its activity.

In vivo activity. Materials and methods. Sterile physiologic salt solution was used for diluting the stock suspension of fungicidin. All tests were made in white mice (Albany strain) of 20-25 grams. Mice were infected by intraperitoneal and intravenous injections of saline suspensions of *C. neoformans* or intraperitoneal injections of *Histoplasma capsulatum.*

Results: Toxicity. The approximate LD 50 of crude fungicidin administered intraperitoneally to mice is between 20 and 26 mg/kg. Injected subcutaneously, however, 2 g/kg did not kill. There was induration and necrosis at the site of injection. No evidence of necrosis was found in the 4 mice receiving 473 mg/kg. Mice receiving a total of 735 mg/kg over a period of 4 weeks showed no ill effects; the fungicidin was administered subcutaneously or intraperitoneally in doses of 200-400 µg, 5 days the first week, 5 days the second week, 4 days the third week, and 3 days the fourth week. In appearance and weight they compared favorably with the control untreated group.

Therapeutic activity. While the data available on the therapeutic activity of fungicidin are limited, Tables III and IV present definite evidence that mice injected with large amounts of *C. neoformans* or *H. capsulatum* and given repeated parenteral doses of fungicidin, have a milder form of infection and life is prolonged beyond that of the controls.

Summary. Two antibiotics of different chemical and biologic properties have been recovered from a soil actinomycete, *Streptomyces* sp.). The one is extracellular and

TABLE IV. Therapeutic Activity of Fungicidin in Mice Infected with *H. capsulatum.*

Infection		Therapy				% survival weeks				
Date	No.* of microorganisms	Dates	No. of inj.	Total amt. inj. mg/kg	No. of mice, 20 g	1	2	3	4	5
10/30/50	6,000,000,000	10/30/50 through 12/4/50	15†	1300	10	80‡	80	80	80	80
10/30/50	6,000,000,000		0	0	10	100	50	0	0	0

* Determined by hemocytometer.

† Mice received 9 inj. the first week, 2 the second, 1 the third, 2 the fourth, and 1 the fifth.

‡ 2 mice died within 48 hr after infection. They were thought to have been injured at time of treatment.

Infection by intraperitoneal route. Therapy by subcutaneous route. Doses ranged from 1-2 mg per mouse.

resembles actidione. The other, designated as fungicidin, is intracellular. It is both fungistatic and fungicidal and apparently lacks antibacterial action. Its activity is not diminished by the presence of horse blood or serum. Fungicidin is distinguished from other antibiotics within our knowledge by its antimicrobial spectrum and its solubility characteristics. The approximate LD 50 for crude fungicidin administered intraperitoneally to mice is between 20 and 26 mg/kg. Injected subcutaneously, its toxicity is considerably lower. Therapeutically, fungicidin appears to be of value in histoplasmosis and cryptococcosis induced in mice.

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Concentration and Distribution of "Encephalin" in the Brain of Humans and Animals. (18398)

W. RAAB AND W. GIGEE.

From the Division of Experimental Medicine, University of Vermont, College of Medicine, Burlington, Vt.

The term "encephalin" designates a substance (or compound of substances?) with sympathomimetic biological action, which has been isolated from human and animal brains (1). It possesses certain epinephrine-like chromogenic and adsorbability properties, but differs from both epinephrine and nor-epinephrine in several respects. The colorimetric readings, obtained in concentrated brain dialyzates against an epinephrine standard were generally in satisfactory quantitative agreement with the pharmacodynamic action of the dialyzates (cat blood pressure, rabbit intestine). Small amounts of nor-epinephrine and epinephrine which have recently been demonstrated in brain extracts and which are believed to derive from sympathetic nerve endings in the cerebral vessels(2) are undoubtedly also present in the encephalin-containing brain dialyzates. Their calculated chromogenic effect would account for only about 1/60 of the total colorimetric readings. Various observations speak against the assumption(2) that the bulk of encephalin might be identical with tyramine. They have been discussed elsewhere(1). One of them is the fact that tyramine does not give the blue color reaction with arsenomolybdic acid which encephalin shares with the known sympathomimetic catecholamines.

Colorimetric assays of encephalin, the re-

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TABLE I. Average Concentrations of "Encephalin" per g (or cc) Expressed in Gamma-Equivalents (Colorimetric Epinephrine Standards).

	Rat (271)*	Rabbit (5)	Dog (5)	Cow (15)	Bull (9)	Hog (7)	Monkey (5)	Man (16)	Human infant (6)
Brain (total)	2.8	1.2	2.0	1.2	1.2	0.8	1.7	1.0	1.5
Cortex	—	—	1.7	1.5	1.2	0.8	1.9	1.2	1.9
Corpus callosum	—	—	1.1	0.9	0.9	0.8	1.0	0.7	1.5
Thalamus	—	—	—	0.8	0.9	0.7	2.1	1.2	1.5
Nucl-caudatus	—	—	3.2	2.9	4.5	2.2	5.0	1.9	2.4
Cerebellum (2)*	—	—	—	1.7	—	—	—	1.5	—
Plexus chorioideus (4)	—	—	—	1.3	—	—	—	1.3	2.2
Pituitary gland (4)	—	—	—	—	—	—	—	3.6	—
Cisternal fluid (18)	—	—	—	—	—	—	—	0.4	—
Lumbar fluid (30)	—	—	—	—	—	—	—	0.2	—

* The figures in brackets indicate the numbers of specimens examined.

sults of which are reported in the following, were carried out in different areas of the brains of humans and of several species of larger mammals, and furthermore, in the mashed whole brains of white rats under a variety of experimental conditions. Shaw's colorimetric method, as modified by one of us (3) and as adapted for brain tissue(1) was used throughout. For each experimental rat brain, a corresponding control specimen was analyzed on the same day with the same reagents. A total of 620 brains was examined.

Results. As shown in Table I, the highest encephalin concentrations were found in the nucleus caudatus, the lowest in the white matter (corpus callosum), with the cortex and thalamus values in between. The encephalin concentration in the nucleus caudatus of the bull was considerably higher than in that of the cow and highest of all in that of the rhesus monkey. The total brain concentration of encephalin was highest in the rat and dog, lowest in the hog and human, and higher in babies than in adult humans. Positive colorimetric readings were also obtained from the cerebellum, chorioid plexus, pituitary gland, and cerebrospinal fluid. The readings in the cisternal fluid were twice as high as those in the lumbar fluid.

The total encephalin content and its distribution were practically identical in two groups of human brains, which were somewhat arbitrarily designated as "normal" (16 specimens) and as "psychotic" (11 specimens). The average values for frontal cortex, motor cortex, corpus callosum, thalamus, nucleus caudatus and whole brain did not differ more than 0–10% between the two groups.

No major changes in the encephalin content of the brain of the rat (average 22 specimens per group; encephalin deviations from –13 to +15%) were found under the following experimental conditions:

(1) Epinephrine (10-40 mg/kg i.p.); all animals died within 5-48 minutes; (2) norepinephrine (40-60 mg/kg i.p.; the chromogenic intensity of nor-epinephrine is about 1/3 of that of epinephrine); died or killed within 3-20 minutes; (3) hydroxytyramine (400 mg/kg i.p.; the chromogenic effect of hydroxytyramine is about 1/70 of that of epinephrine); all killed after 30 minutes; (4) desoxycorticosterone acetate (2.5 mg i.m. daily on 8 successive days); all killed; (5) thyroxine (1 mg i.p. on 3 successive days); all killed; (6) insulin (10-40 units i.p.); all killed after 60 minutes; a second group received 20 units on 2-3 successive days; all killed 4-24 hours after last injection; (7) adrenalectomy; died or killed within 2-50 (average 19) days; (8) hypophysectomy; all killed within 82-95 days.

(9) Nembutal (6-18 mg i.p.); died or killed after 15-30 minutes; (10) morphine sulphate (20-120 mg i.p.); died or killed after 16-18 minutes.

(11) Benzedrine (10 mg i.p.); convulsions, all killed after 60 minutes; (12) metrazol (20 mg i.p.); convulsions, died or killed after 2-9 minutes; (13) strychnine (1.5 mg i.p.); convulsions, died or killed after 2-5 minutes;

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3. Raab, W., *Exp. Med. and Surg.*, 1943, v1, 188.

(14) DC current through the head (10 seconds to 5 minutes); died or killed; (15) AC current through the head (Variac positions 6-32, 15-165 minutes); all died.

(16) Excitement (tied on back for 4 hours); all killed; (17) exercise (swimming in water, 30°C, 30-60 minutes); all killed; (18) low air pressure (252 mm Hg, 4 hours); all killed; (19) cold exposure (–10 to +6°C, 3-24 hours); all killed; (20) acetylcholine (under nembutal anesthesia, 15 mg in 0.2 to 0.3 cc of Ringer solution injected into a carotid artery craniad); died or killed after 1-5 minutes; (21) alcohol (daily 4 cc of absolute alcohol ingested with drinking water); died or killed after 8-24 (average 25) days; (22) N-free diet (obtained from General Biochemicals, Inc.); all killed after 23-43 days.

Highly significant changes of the colorimetric readings were observed only after i.p. injection of dihydroxyphenylalanine (300 mg/kg), namely a rise of 120% after 30 minutes, of 134% after 60 minutes, and of 66% after 120 minutes. (The chromogenic intensity of dihydroxyphenylalanine is about 1/3 of that of epinephrine.) All animals were killed.

In some of the rats which received epinephrine, hydroxytyramine or dihydroxyphenylalanine, the heart was analyzed and an accumulation of these substances was clearly demonstrable in accordance with their respective chromogenic intensity and dosage.

Comment. As long as nothing is known concerning the possible role of encephalin in cerebral metabolism, it would be futile to speculate on the functional significance of the pattern of its intracerebral distribution, which proved to be essentially the same in all species examined, except that in adult humans the average differences between the cortex and the brain stem ganglia were smaller than in animals. It is noteworthy that the colorimetric encephalin concentration in rabbit and cattle brain is about the same (1.1 and 1.2 γ equivalents per gram, respectively), whereas the concentration of nor-epinephrine-epinephrine has been found about 10 times higher in the brain of the rabbit than in cattle brain (0.2 γ per gram vs. 0.02 γ per gram)(2). The presence in the chorioid plexus, the pituitary gland, and the cerebrospinal fluid of material with the same adsorbability and chromogenic properties as encephalin suggests the possibility of neurosecretory discharges of encephalin from the brain into adjacent structures. In this connection, the question arises as to whether encephalin might play a similar part in the mechanism of ACTH secretion as epinephrine(4,5). The lower chromogenicity of the lumbar fluid, compared with the cisternal fluid, makes it probable that encephalin diffuses out of the spinal canal into the general circulation. Incubation of cisternal fluid at 37°C for 8 hours did not alter its colorimetric readings. The absence of any appreciable difference between the quantity and distribution of encephalin in the brains of "normal" and "psychotic" persons, although to be regarded with caution because of the somewhat arbitrary selection of cases, seems to make it unlikely that quantitative alterations of encephalin in the brain are a factor in mental disease.

The over-all concentration of encephalin in the brain of the rat proved remarkably constant under a variety of experimental procedures, such as injection of hormones, removal of endocrine glands, administration of centrally stimulating and depressing drugs, stressful situations, injection of acetylcholine, and N-free diet. The conclusion appears justified that the formation of encephalin is not dependent upon the integrity of the endocrine system, especially the adrenal glands, and that convulsions, general anesthesia, etc., occur without any gross quantitative changes of the encephalin content of the brain. However, this does not rule out the possibility of qualitative alterations of the molecular structure of encephalin which might escape detection by colorimetry. In contrast to the heart muscle, which eagerly accumulates sympathomimetic amines in large quantities, the rat brain did not retain either epinephrine, norepinephrine, or hydroxytyramine. On the

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other hand, injection of dihydroxyphenylalanine (Dopa) was followed by a very marked increase of chromogenicity of the brain extracts which reached its maximum within one hour and was on the decline after two hours. Whether this phenomenon is due to mere temporary deposition of Dopa in the brain or to a conversion into encephalin, is a question which deserves further study with the aid of paper chromatography.

Summary. Colorimetric assay of a large number of human and animal brains for "encephalin" revealed a rather uniform pattern of intracerebral distribution in various species. There was no appreciable difference between the encephalin content and its distribution in the brains of "normal" and "psychotic" humans. No major changes were observed under the influence of various hormones, adrenalectomy, hypophysectomy, convulsive drugs, electrical stimulation of the brain, narcotics, conditions of stress, acetylcholine, and N-free diet. Only the injection of dihydroxyphenylalanine was followed by a very marked temporary increase of the colorimetric readings, which suggests either a selective absorption of this substance by the brain or its conversion into encephalin.

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Metabolism of Isomers of Linoleic and Linolenic Acids.* (18399)

Ralph T. Holman. (Introduced by Carl M. Lyman.)

From the Department of Biochemistry and Nutrition, Texas Agricultural Experiment Station, Texas Agricultural and Mechanical College System, College Station, Texas.

It has been repeatedly shown that the rigid exclusion of fat from the diet of rats and other animals results in cessation of growth and appearance of skin symptoms which are prevented or cured by either linoleic or arachidonic acid(1). However, either linolenic acid or hexaenoic acid permits growth, but does not cure the skin symptoms. The administration of linoleate to fat deficient rats causes the deposition of predominantly arachidonic acid (tetraenoate), and the administration of linolenic acid causes deposition of predominantly hexaenoate in certain tissues(2). However, the conversion of linoleate to hexaenoate and linolenate to tetraenoate has been demonstrated in the whole animal. Reiser has recently shown the conversion of linoleate to tetraenoate and pentaenoate, and of linolenate to hexaenoate in the chick(3). To attempt to learn something of the nature of the highly unsaturated acids deposited as the result of linoleate or linolenate feeding, a study was made of the deposition of polyunsaturated fatty acids in the rat as the result of administration of unnatural isomers of the essential fatty acids.

Experimental. Rats which had been kept on a fat deficient diet(2) for 10-12 months after weaning were given daily oral doses of 40 mg of ethyl esters of the various fatty acids under study for a period of 2 months. At the end of this time, the animals were killed, the total fatty acids were isolated, the polyunsaturates were estimated spectrophotometrically(4), and the total quantities of 4, 5 and 6 double bond acids were estimated as described previously(2). Ethyl linoleate and ethyl linolenate were prepared by the usual bromination-debromination procedures. Ethyl linolelaidate was prepared by selenium iso-

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TABLE I. Deposition of Polyunsaturated Fatty Acids as the Result of Supplementation.

Supplement	Animals	Avg wt gain, g	Total fatty acids, g/rat	Hexaenoic acid, mg/rat	Pentaenoic acid, mg/rat	Tetraenoic acid, mg/rat
None	2	0	3.2	35		9
Linoleate	4	96	10.4	155		78
Linolelaidate	5	2	4.4	67		27
Conj. linoleate	2	—36	0.5	15		7
Linolenate	4	69	7.2	164	22	88
Elaidolinolenate	3	33	13.8	288		
Arachidonate(10)	4	77	8.3	126	8	217

merization of linoleate(5). Ethyl elaidolinolenate was prepared by the method of Kass, Nichols and Burr(6). Conjugated linoleic acid, (m.p. 8°), was prepared from linoleic acid by alkaline isomerization(7).

Results of the investigation, shown in Table I, indicate that both linoleate and linolenate cause increases in total tetraenoate and hexaenoate in the whole animal. These results at first glance disagree with earlier conclusions (2,8) which were based on analyses of selected tissues, and in which the conversions of linoleate to hexaenoate and linolenate to tetraenoate escaped notice. The present data, however, in partial agreement with Reiser's data obtained on chickens(3) raise the question why tetraenoate formed from linolenate is inactive in curing the skin symptoms of fat deficiency.

Trans isomers of linoleate and linolenate, although not active in alleviating fat deficiency symptoms, are converted to spectrophotometrically detectable polyunsaturated forms. Linolelaidate (*trans, trans*-linoleate) is converted to both tetraenoate and hexaenoate, but to a lesser degree than is natural linoleate. Likewise, elaidolinolenate (*trans, trans, trans*-linolenate) is converted to hexaenoate and pentaenoate. However, these polyunsaturated fatty acids formed from the unnatural isomers of linoleate and linolenate are probably themselves unnatural isomers containing *trans* double bonds, which although detectable by alkaline isomerization and spectrophotometric technic, are biologically inactive forms.

Conjugated linoleic acid is not converted to polyunsaturated acids. Its administration caused a decreased weight and the early death of the rats. Upon analysis, the polyunsaturated acid content of the animals was found to be less than in the deficient control animals. In as much as conjugated acids oxidize by a different mechanism than do the methylene-interrupted polyunsaturated acids (9), it would be expected that metabolism of conjugated linoleic acid would also be different. The possibility exists that the metabolic oxidation of conjugated linoleic acid has a wasting effect upon the structural polyunsaturated acids of the animal.

In these experiments classical symptoms of fat deficiency were cured only by ethyl linoleate. Arachidonate, fed in earlier parallel experiments(10) also cured symptoms and caused renewed growth. Data from the arachidonate experiment are included in Table I for comparison. Conjugated linoleic acid worsened the fat deficiency symptoms. Weight gains during the 2 months supplementation period were good for linoleate and linolenate. For linolelaidate no gain was made and for elaidolinolenate only a moderate gain was observed. Supplementation with linoleate, linolenate or elaidolinolenate caused significant increases in the total fatty acids of the rat, but the increases as the result of linolelaidate feeding were somewhat less. Feeding conjugated linoleic acid drastically

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reduced the total fatty acids of the animals.

Summary. From these experiments it is apparent that *trans* isomers of the "essential" fatty acids are converted to inactive forms of polyunsaturated acids which are, however, detectable spectrophotometrically. Conversely, the detection of polyunsaturated acid in a tissue does not imply that these substances are biologically active. However, this factor is not likely to affect studies of animals fed natural diets.

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The Effect of Acclimatization on the Propulsive Motility of the Small Intestine During Anoxia.* (18400)

EDWARD J. VAN LIERE, J. CLIFFORD STICKNEY, AND DAVID W. NORTHUP.
From the Department of Physiology, School of Medicine, West Virginia University, Morgantown

Only a relatively few objective criteria have been established for adaptation to oxygen want. Perhaps the two most widely recognized are the increases in the number of red blood cells and in the amount of hemoglobin. It is desirable to establish other criteria and it is always appropriate to study adaptation in different species of animals. In the present study albino rats were used.

We have shown(1) that the propulsive motility of the small intestine of rats is decreased by effective degrees of hypoxia. Actually a pressure of only 382 mm of Hg (simulated altitude of 18,000 ft.) is capable of producing a significant decrease of motility. If rats became acclimatized to a low oxygen tension, and then were exposed to altitude, the propulsive motility should not be affected to the same degree as it would in unacclimatized animals.

Methods. Seventeen albino rats (Wistar-strain) were acclimatized by placing them in a low-pressure chamber for three and one-half hours each day at a pressure of 303 mm Hg (simulated altitude of 24,000 ft.); 8 rats were exposed for 26 and 9 for 62 days. The temperature of the room in which the low-pressure chamber was located varied from 70° to 75° F. Forty-eight hours after the last acclimatization period the rats were paired with normal unacclimatized rats, which were similar in weight and age. They had been fasted for 24 hours. Each member of a pair of these rats was given 2 cc of a mixture of 10% powdered charcoal suspension in 10% gum acacia in water by stomach tube. After allowing 10 minutes for some of the material to enter the small intestine, both rats were placed in the low-pressure chamber at a pressure of 254 mm Hg (simulated altitude of 28,000 ft.) At the end of 30 minutes they were taken out of the chamber and decapitated. The small intestine was removed, slit open and the distance the charcoal had traversed measured.

In order to ascertain whether acclimatization alone affects normal propulsive motility of the small intestine 11 rats were acclimatized using the same general procedure as previously outlined. They were exposed to a low oxygen tension for four hours each day for 41 days. They were then paired with unacclimatized rats and the propulsive motility of the small intestine determined (at ground level) in the usual manner. No significant difference was found.

Results. The results are given in Table I. The latter shows that the propulsive motility of the small intestine of the acclimatized rats was less affected by hypoxia than the unacclimatized. The results were statistically significant at the 0.1% level (Fisher's t test). It was found, moreover, that those animals which had been exposed to hypoxia for 3.6 hours each day for 26 days were as well

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* Aided by a Grant of the Ella Sachs Plotz Foundation.

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TABLE I. Effect of Anoxia on Rats Acclimatized to Altitude of 24,000 Ft. (3.6 hr each day for 62 days).

Unacclimatized				Acclimatized			
Body wt, g	Length of intestine, cm	Length traversed		Body wt, g	Length of intestine, cm	Length traversed	
		cm	%			cm	%
330	125	41	33	440	110	42	38
340	111	31	28	390	123	44	36
330	122	42	34	370	112	68	61
320	110	47	43	360	116	80	69
310	109	44	40	310	117	69	59
310	115	47	41	340	111	62	56
300	119	57	48	290	116	79	68
290	111	46	41	280	117	64	55
290	114	41	36	220	111	75	68
Avg 313.3	115.1	44.0*	38.2†	333.3	114.8	64.8*	56.7†

* t = 4.07942; P = <.001.
† t = 4.15579; P = <.001.

acclimatized—as far as the response of the intestine to hypoxia was concerned—as those which had been exposed for the same number of hours each day for 62 days.

Discussion. That the propulsive motility of the small intestine of acclimatized rats was less affected by hypoxia than that of the unacclimatized animals was not unexpected. We have shown previously (2) that gastric emptying in the dog, which is known to be delayed by oxygen want, will return to normal at altitude following effective acclimatization. It appears, then, that in the acclimated rat the propulsive motility of the small intestine tends also to regain its normal activity under conditions of low oxygen tension. Dogs, incidentally, could not be used in this current study, for we have shown that the propulsive motility of the small intestine of the adult dog is unaffected even by severe degrees of hypoxia(3). It is worthwhile to study the effects of acclimatization to altitude on separate organs since some of them respond more readily to adaptation than others. Data obtained, furthermore, from such studies throw light on the complicated processes known to be associated with the adaptation of an organism to altitude.

Summary. The propulsive motility of the small intestine of rats, which had been acclimatized to hypoxia, was significantly less affected at altitude than were unacclimatized animals. This was interpreted to mean that the effect of hypoxia on the propulsive motility of the small intestine could be used as another criterion for acclimatization.

Thanks are due to Helen Hope Hess for acclimatizing one group of rats.

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Conversion of Caproic Acid to Acetoacetic Acid in the Goat.* (18401)

G. W. E. PLAUT† AND VEARL R. SMITH.

From the Departments of Biochemistry and Dairy Husbandry, College of Agriculture, University of Wisconsin, Madison.

Ketosis is a common metabolic disorder of ruminants which is of considerable economic importance. In ketosis of dairy cattle, the blood composition is altered by a marked decrease in blood glucose and an increase in ketone bodies. MacKay *et al.*(1) reported that saturated fatty acids with 4, 6, 8 and 10 carbon atoms were ketogenic when fed to normal rats receiving adequate amounts of carbohydrates. Oral administration to goats of single doses of butyric, caproic, caprylic or capric acid has been shown to cause an increase of the total blood ketone level of 5 to 10 mg % [Schultz, Smith and Lardy(2)]. Maximum concentrations of the ketone bodies were attained 15 minutes after administration of butyric acid and 1 hour after the administration of caproic, caprylic or capric acid. It was not clear from that study whether these acids are converted directly to ketone bodies or merely stimulate their production.

Methods. 10.6 g of caproic acid-l-C^{14} containing 6.9×10^8 c.p.m. in 20 cc of water was administered orally by means of a stomach tube to a normal fed mature female goat weighing 107 lb. Blood samples were taken from the jugular vein before administration of the fatty acid and at 15, 30, 60 and 90 minutes thereafter. Perchloric acid was added to a final concentration of 4.5% to deproteinize the blood. Acetoacetic acid was determined on an aliquot of the blood by the method of Behre and Benedict(3). 0.625 mM of acetoacetate per 100 ml of deproteinized filtrate was added as a carrier and acetoacetic acid was degraded as described by Plaut and Lardy(4). All samples of barium carbonate and acetone 2,4-dinitrophenylhydrazone were reprecipitated to constant radioactivity. The substances were plated on aluminum dishes and counted with a Q-gas counter. The counts were corrected for background and self absorption [Calvin *et al.*(5)].

Results and discussion. Fifteen minutes after the administration of the radioactive caproate a rise in the acetoacetate content of the blood was observed. Caproate appears to be converted to acetoacetate as evidenced by the parallelism of the concentration and the radioactivity of this ketone body in the blood (Table I). These results may be of significance in the development of ketosis when considered in view of the recently acquired knowledge of rumen digestion (see Phillipson for review)(6). The short chain fatty acids, acetic, propionic and butyric, are important products of rumen fermentation and are absorbed directly into the blood. Acetic and propionic acids, which are produced in the rumen in greater quantities than butyric, do not cause an appreciable increase in blood ketone levels when fed orally to goats (Schultz, Smith and Lardy)(2). In fact, the intravenous injection of propionate into sheep has been reported (Jarrett and Potter)(7) to produce a fall in blood ketone bodies. Therefore, should conditions arise

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† Predoctoral Fellow, National Cancer Institute.

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5. Calvin, M., Heidelberger, C., Reed, J. C., Tolbert, B. M., and Yankwich, P. E., *Isotopic Carbon*, New York, 1949.

6. Phillipson, A. T., *Nutrition Abstracts and Rev.*, 1947, v17, 12.

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TABLE I. Incorporation of Carboxyl Carbon of Caproate Into Blood Acetoacetate.

Time of blood withdrawal after admin. of of caproate-1-C^{14}, min.	Acetoacetate, μM/ml of blood	Radioactivity of acetoacetate in presence of 'carrier' as c.p.m. per mM			Ratio of radioactivity Carbons 2,3,4 Carbon 1, avg
		Carbon 1	Carbon 2,3,4	Total acetoacetate	
0	22				
15	166	10500 ± 200	3650 ± 65	14060 ± 210	.34
30	110	3920 ± 80	1330 ± 40	5250 ± 90	.34
60	103	3980 ± 70	770 ± 25	4750 ± 75	.19
90	100	2240 ± 55	650 ± 30	2890 ± 63	.29

which would alter the fermentation in the rumen, greater quantities of butyric or longer chain fatty acids might be produced. If this should occur, the acids produced in the rumen might be a factor in the development of ketosis.

It is of interest that the activity of the acetone portion and the carboxyl group of the acetoacetate is not equal, which points to the possibility that when caproate is administered to the whole animal, the recondensation of two-carbon fragments derived from this fatty acid does not occur in a random fashion. This is in accord with the observations of Crandall *et al.*(8,9), in their experiments on the metabolism of fatty acids by washed rat liver homogenates.

Summary. Caproate 1-C^{14} was administered to a goat. The resulting increase of blood acetoacetate is roughly proportional to the rise in radioactivity of this compound. The specific radioactivity per mole of the carboxyl group of the acetoacetate is larger than that of the acetone portion. This is another indication that two-carbon compounds derived from fatty acid do not condense in a random fashion to form acetoacetate.

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Effect of Colchicine on Tumor Growth and Tumor Pyrophosphatase. (18402)

L. BLOCH-FRANKENTHAL AND A. BACK.
(Introduced by B. Zondek.)
From the Department of Radiology, Cancer Laboratories, The Hebrew University-Hadassah Medical School, Jerusalem, Israel.

A new attempt has recently been made to explain the influence of colchicine on tumors. According to this theory the regression of tumors obtained by injections of colchicine is due to the action of this substance on the endothelial cells of growing capillaries of the tumor. This action was very marked in "highly cellular rapidly growing tumors"(1). This explanation compares the action of colchicine to that of polysaccharides obtained from *Seratia marcenses* [Shear and coworkers (2,3)]. In the strain of tumor with which we worked, we were unable to observe this hemorrhagic action of colchicine, and since we found this tumor to contain a highly active enzyme, capable of hydrolysing sodium pyrophosphate, we deemed it worthwhile to examine the dependence of this enzyme system

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on the colchicine injections.

In this paper we describe the influence of colchicine on the growth of a benzopyrene-induced sarcoma (tumor HL) and on the pyrophosphatase contained in it.

Methods. (a) *Rats.* We employed albino rats of both sexes weighing 150 to 175 g, bred in our laboratory for more than 7 years through brother-sister cross-mating. The animals were fed Purina Chow and water *ad libitum*.

(b) *Tumor.* The tumor employed in these experiments was a benzopyrene-induced sarcoma obtained in 1936, and transferred from rat to rat by subcutaneous implantation in the dorsal region. Histologically this tumor is a sarcoma of conjunctival origin with a majority of fusiform cells and containing other cells of different forms and dimensions varying from fusiform cells to giant polynuclear cells. The tumor contains very few blood vessels and is of a hard consistency. In the first stage of their development the tumors were designated as "lentil-", "pea-" and "bean"-sized because exact measurements were difficult to carry out. In the following stages the tumor was measured with a caliper. Each growth experiment lasted 6 to 8 weeks, depending on the cachectic state of the control animals. At the end of each experiment the tumors were excised, measured and weighed.

(c) *Colchicine.* We used BDH crist. puriss. Colchicine in a 0.05% solution in distilled water. Each solution was used for no more than a week after preparation and was maintained at 4° C when not in use. The injections were made subcutaneously in the vicinity of the tumor but not into it. In one series of experiments the injections were begun 3 days after the inoculation of the tumor, but in the majority of cases, 5 to 6 days after tumor inoculation. This period is roughly half the time required by a tumor to reach the size of a lentil, when it first becomes readily observable. In some cases the injections were begun at the lentil stage.

(d) *Pyrophosphatase estimation.* Tumor tissue obtained from rats anesthetised with ether was freed as far as possible from foreign matter such as blood, etc. and suspended in water with the aid of a Potter-ground glass homogeniser. The suspension obtained was tested, usually at 2 concentrations, as to its activity on sodium pyrophosphate under the following conditions:

(1) 1 cc tumor suspension + 2.5 cc acetate-veronal buffer (pH-6.8) + 1 cc $MgCl_2$ 1% ($MgCl_2 \cdot 6H_2O$) + 0.2 cc sodium pyrophosphate 1% ($Na_4P_2O_7 \cdot 10H_2O$) + H_2O to a volume of 5 cc.

(2) Same as in (1) with half the concentration of suspension. The mixtures were incubated at 37° C and the course of hydrolysis was followed by estimating the liberated phosphate after stipulated time intervals. Usually two determinations were carried out, one after 30 minutes' incubation and another

TABLE I. Effect of Colchicine Injections on the Tumor Growth in Grams. Colchicine treatment in all series was identical.

Series No.	Rat No.	Wt of tumor in g	Rat No.	Wt of tumor in g
Colchicine treated rats (experiments)			Untreated rats (controls)	
1	1	.0		
	2	7.4	10	65
	3	.3		
	4	.2	11	105
	5	10.0		
	8	7.5	12	50
2	16	.2		
	18	5.5	19	38.5
	21	9.0		
	23	.1	20	35
3	24	.0		
	25	19.5	34	51
	26	.5		
	28	9.5	35	38.5
	29	31.5		
	31	.3	36	80
	32	13.5		
	33	17.2	39	82
4	38	1.6		
	40	.7		
	43	36.2	47	62.5
	44	1.7		
	45	.0	48	31
	46	.7		
	50	4.8	49	45
	51	2.8		
	54	.0		
5	101	7.2	97	47.5
	102	.1	98	58.5
	103	1.2	99	85
	118	33.0	100	61
	119	.0	123	40
			125	65
			126	65
			127	87

TABLE II. Effect of Colchicine Treatment of Pyrophosphatase Activity of the Tumor.

Rat No.	0.05% colchicine admin., cc	Period of colchicine treatment	Conc. of tumor suspension tested	Pyrophosphate-hydrolysed after		Pyrophosphate hydrolysed by control tumor suspension of untreated rat after	
				30 min., %	120 min., %	30 min., %	120 min., %
108	1.6	7 days	1/10	16	30	100	
106	1.9	13 ”	1/50	15		50	
107	1.6	20 ”	1/50	14		50	
115	0.9	5 ”	1/10	12		100	
132	1.7	12 ”	1/20	10	12	100	
135	1	9 ”	1/40	4.5	7.2	65	100
157	1.4	12 ”	1/40	16.5	34	90	100
164	2.7	20 ”	1/40	14	26	73	100
176	1.8	10 ”	1/40	5	12.5	100	
178	2	11 ”	1/80	11	21	32.6	70.3
180	1.9	10 ”	1/40	7.8	17.7	80	100
181	1.9	10 ”	1/40	7	20	80	100
175	2.2	10 ”	1/40	42		74.5	
183	3.2	16 ”	1/40	11	18	100	
148	1.6	14 ”	1/40 1/80	No depression in pyrophosphatase activity was observed in these cases			
149	1.8	15 ”	1/40 1/80				
150	1.8	15 ”	1/40 1/80				
158	1.4	12 ”	1/40 1/80				
163	1.6	12 ”	1/40 1/80				
165	2.7	20 ”	1/40 1/80				
172	2	9 ”	1/40 1/80				

after 2 hours. Phosphate determinations were carried out according to the method of Fiske and Subarow as modified by Lohman and Jendrassik(4) with the aid of a "Lumetron" photometer.

Controls of enzyme, alone, were carried out in each case. Pyrophosphate alone did not liberate any phosphate under the conditions of our experiments. The results, (Table III), represent the corrected values of free phosphate found expressed as percent of the total hydrolisable phosphate present in the mixture.

Results and discussion. As may be seen from Fig. 1 and Table I and II, treatment with Colchicine in most cases caused a considerable deceleration in the growth of tumors. Fig. 1 gives a description of samples from 4 series of experiments. The times of the initiation of colchicine injections fell into 3 groups. In Series 1 the injections were begun 4 days after inoculation, in Series 2 and 3, 5 days after inoculation, roughly half the period required by a tumor to reach the size of a lentil. In the 4th series the injections were begun as late as 14 days after inoculation of the tumor, when it had reached the "lentil" stage. No differences in effect were found among these series. The quantity of colchicine and its distribution in time was also varied from series to series, but was equal in all experiments of a single series.

Table I summarizes increases in weight of tumors in colchicine-treated rats as compared with controls. The difference in weight between these 2 groups is clear. It may be added that the weight of the 3 smallest control tumors is approximately equal to the maximum of 3 tumors treated with colchicine, and that the maximum weight of controls is about 3 times that of the treated ones. However, in each series there were some tumors which were relatively resistant to the action of colchicine.

We were able to observe that in many points the reaction of the strain with which we were working was similar to that described previously by Ludford and others. We could not find any case of hemorrhage in our treated tumors, and an assumption that the action of colchicine in the case of this tumor strain may be analogous to that of bacterial polysaccharide is not probable. As to the

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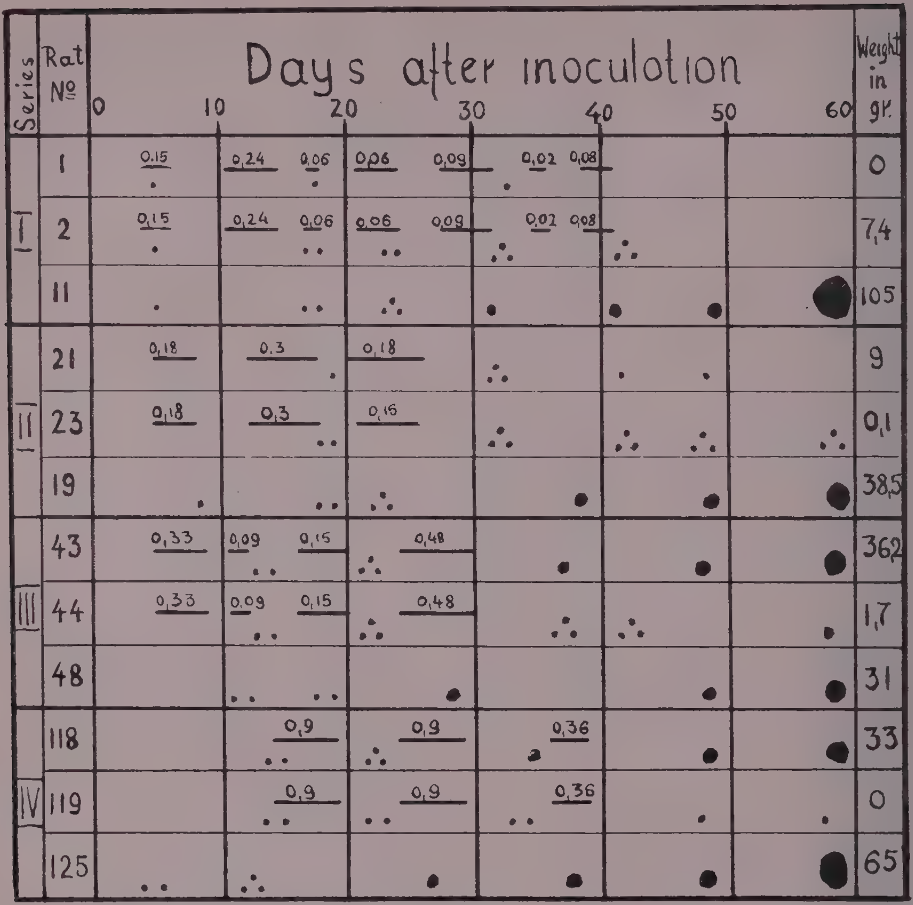

FIG. 1.

The course of some typical experiments from 4 different series on the effect of colchicine on growth. In each series one control and 2 colchicine experiments are presented.

• — lentil size; • • — pea size; ∴ — bean size.

The denotation of bigger sizes is arbitrary, but each sign is proportional to actual size. Numbers in table denote mgs of colchicine administered. Length of the line under each number is proportional to the number of days this quantity of colchicine was administered.

effect of colchicine on pyrophosphatase, it has already been mentioned that our tumor (HL) was found to contain a potent pyrophosphatatic agent. Its pH optimum was found to be in the range of neutrality in the presence of $MgCl_2$. This salt highly activates the pyrophosphatase, but the tumor displays some activity even without the addition of $MgCl_2$, in contrast to erythrocytes* and brain tissue(6), both of which have about the same pH optimum with regard to pyrophosphate hydrolysis but are almost inactive without added $MgCl_2$.

Among the rat organs tested under the above mentioned conditions (pH 6.8 with added $MgCl_2$) tumor HL was found to be one of the tissues richest in pyrophosphatase. Liver heads the list, and is followed by kidney and tumor HL, both of which are of the same order of activity. These are followed by erythrocytes, muscle and dermis in the order mentioned. It should be added that the latter is the mother tissue of tumor HL, but its activity amounts to only about one-fourth to one-third of that of the tumor tissue.

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* According to our own experience as well as that of Nagana(5).

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The pyrophosphatase experiments were carried out on a separate batch of rats. Colchicine injections for the purpose of investigating the effect of this drug on the pyrophosphatase activity were administered during the last 2 or 3 weeks before the termination of the experiments. The injections were administered at various stages of tumor growth, and 0.1 mg to 0.15 mg were given daily as often as the rats' state of health permitted. Colchicine injections generally induced diarrhea. On the average the total dose they received amounted to 1 to 1.5 mg.

It should also be mentioned that a high pyrophosphatatic activity was found only in healthy tumor tissue, and that necrotic tissue is practically inactive.

Colchicine has no effect whatever on the pyrophosphatatic activity of tumor tissue *in vitro,* when added to the reaction mixture itself or when incubated with the tissue prior to the addition of the other ingredients. Previous incubation of the tissue causes a decrease in activity, but this is not influenced by the presence of colchicine.

Colchicine *in vivo, i.e.* colchicine administered to the tumor-bearing rat subcutaneously in the vicinity of the tumor for some period of time, had an inhibiting effect on the pyrophosphatase activity in most of the cases tested (Table II). This effect could not be observed in any of the other tissues tested, namely liver, kidney, muscle or skin. In the case of the last 2 tissues the material was taken from the area in which colchicine injections were administered[†]. The effect does not seem to be a local one.

An acceleration is observed in erythrocytes after colchicine injections, but this is due to the higher erythrocyte concentration in the blood of rats treated with colchicine, caused by the diarrhea and loss of water. On adjusting the erythrocyte concentration of the blood to equal that of the control, equal activities were obtained with normal and colchicine treated rats.[‡]

In conclusion it may be added that since on the one hand colchicine inhibits the growth of tumor HL in which case no hemorrhage was observed and reduces one of its enzyme activities, and since the rats acquire some tolerance to colchicine after having undergone the first few injections according to our own observations and to those of Ludford, the effect of the latter should further be sought among the enzymatic systems even though the effect on pyrophosphatase does not seem to be the initial one.

Summary. 1. Treatment of a benzopyrene-induced rat sarcoma with colchicine produced tumor regression. This regression was complete in some cases. 2. No hemorrhage has been observed in tumors treated with colchicine. 3. This tumor contains a potent enzyme capable of hydrolysing sodium pyrophosphate. 4. The pyrophosphatase of tumors treated with colchicine is thereby lowered. 5. Colchicine has no effect on tumor pyrophosphatase *in vitro*.

[†] V. S. Waravdekar and J. Leiter observed an analogous effect with peltatins which reduced the oxydase of mouse sarcome 37 whereas no such effect was observed in the liver, spleen or kidneys(7).

7. Waravdekar, W. S., and Leiter, J., *Cancer Research*, 1949, v9, 625.

[‡] Colchicine was injected intravenously for the experiments with erythrocytes.

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Effect of Epinephrine on the Neutrophile Count of Normal and Adrenalectomized Mice.* (18403)

Kathryn F. Stein, Cynthia Martin, and Doris Falk.
From the Department of Zoology, Mount Holyoke College, South Hadley, Mass.

The role of epinephrine in the regulation of the number of leucocytes in the blood and thus in relation to the problem of stress has been discussed by various investigators(1,2). It is well known, and has been confirmed by the present study, that epinephrine injections result in a lymphopenia and an eosinopenia in intact animals and there is also evidence in the literature that this response is prevented by adrenalectomy(3-5). According to Speirs and Meyer(5) the occurrence of an eosinopenia following epinephrine injection in an adrenalectomized animal indicates either incomplete removal of the glands or the presence of accessory adrenal cortical tissue. It is interesting that in the majority of their adrenalectomized mice epinephrine actually caused an eosinophilia. An absolute neutrophilia has also been reported to occur after injection of epinephrine, cortical extracts and following conditions of stress(6,7). According to some investigators adrenalectomy prevents this response of the neutrophiles to epinephrine(8) but this has not been confirmed by later work(9,10) nor by the present study. In the series of experiments reported here it has also been observed that the neutrophilia resulting after epinephrine, both in intact and adrenalectomized mice, was more extreme than that caused by other agents, and an attempt has been made to discover the reason for this difference.

Materials and Methods. The mice used for these experiments came from various sources. Some were albino but the majority were hybrids of unknown ancestry. Many of the latter had been made trichinous by forced feeding with infected muscle. Adrenalectomy or splenectomy were performed on different groups (Table I). In the adrenalectomized series only those individuals which failed to show an eosinopenia after epinephrine injection, and which therefore could be considered completely adrenalectomized(5), were included. Injected substances, in addition to epinephrine or adrenaline, included ACE (lipoadrenal cortex)†, sex hormones (testosterone propionate, estradiol diproprionate and estradiol benzoate) and sesame or peanut oil. Epinephrine was administered either hourly for 4 hours according to the method of Long and Fry(11), or as a single dose; other substances always as single subcutaneous injections. Total white counts were made in the usual manner from tail blood and differentials from smears stained in May-Grunewald-Giemsa. Monocytes were not distinguished from lymphocytes; mononuclears in Table I includes both. One thousand granular leucocytes (all stages) were differentiated as neutrophilic or eosinophilic in bone marrow smears made directly from sternum or femur and stained like the blood smears.

Results. The results (Table I) indicate that the neutrophilia supervening after

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* Aided by a grant from the Committee on Research in Endocrinology, National Research Council.

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† We are indebted to Dr. H. F. Hailman of the Upjohn Company for the Lipo-Adrenal Cortex.

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TABLE I. White Blood Counts of Mice Before and After Injections.

Substance injected	Dosage	No. and type	Time between counts	Avg No. cells before inj. Total	N.	E.	M.	Avg No. cells after inj. Total	N.	E.	M.
			Intact mice.								
ACE in oil (Upjohn)	1/20 cc	8 trich.	4 hr	24800	4960	1240	18600	10600	2540	20	8040
			6 ”					18600	3350	300	14950
	1/10	13 ”	6 ”	17350	4500	1350	11500	9900	3550	100	6250
Epinephrine hydrochloride	0.02 mg 100 g in 4 inj. (hourly)	4 ”	6 ”	19100	5100	2700	11300	18700	7800	400	10500
Adrenaline chloride		13 ”	5 ”	12450	3500	750	8600	17500	10400	350	7200
		5 norm.	5 ”	6700	2300	250	4150	11200	7700	60	3440
	1 dose	3 ”	2 ”	9900	2100	500	7300	10200	7650	200	2350
♀ hormone	25 mg	8 trich.	6 ”	16500	4950	1150	10400	16400	4600	950	10850
Testosterone	70 mg	5 ”	6 ”	19100	3050	1450	14600	18900	3450	1050	14400
Oil	1/10 cc	9 ”	6 ”	16000	4000	1000	11000	17500	4400	900	12200
			Adrenalectomized mice.								
Adrenaline chloride	0.02 mg*	7 ”	5 ”	13000	3100	300	9600	16800	7800	700	8300
	100 g in 4 inj. (hourly)	10 norm.	5 ”	13900	1800	150	11950	16750	4700	350	11700
	1 dose	6 ”	5 ”	9200	1850	150	7200	15600	5700	450	9450
	0.005 mg	4 ”	2 ”	18400	4800	450	13200	18200	6700	1000	10500
	100 g 1 dose		5 ”					26550	7200	1050	18300
			Splenectomized mice.								
Adrenaline chloride	As above*	5 norm.	5 ”	15200	4150	350	9700	16400	9350	50	7000
ACE in oil	1/10 cc	5 ”	6 ”	25500	7400	1500	16600	16400	7000	400	9000

Trich.—trichinous; Norm.—normal; N.—neutrophiles; E. eosinophiles; M.– mononuclears.

epinephrine was greater than that occurring after the other agents tested. This response was present in adrenalectomized as well as in intact animals. Adrenal cortical extract produced a relative though not the absolute neutrophilia noted by Dougherty and White(7). This does not seem explainable on the basis of dosage. Variation in the content of different samples of ACE may possibly be involved, although the 2 different samples utilized for the present study gave markedly uniform results, especially considering the amount of variation in the experimental animals. Though an absolute increase occurred in some individuals after sex hormones the average results, like those after sesame or peanut oil, showed no significant increase.

An attempt has been made to discover the mechanism by which epinephrine exerts its action. Obviously the spleen was not involved since splenectomized animals showed a neutrophilia corresponding to that found in normal and adrenalectomized mice (Table I). It is possible, as suggested by other investigators(12,13) that the effect of epinephrine in increasing the blood flow may serve to send into the actively circulating blood cells which ordinarily remain close to the vessel wall and are not dislodged in the process of taking blood for counts. This might also account for the eosinophilia observed after epinephrine in adrenalectomized animals by Speirs and Meyer(5) and in the present study (Table I). It would indicate the presence peripherally of a larger proportion of polymorphonuclear cells than of lymphocytes, since an absolute lymphocytosis was not found in adrenalectomized animals injected with epinephrine except in one group after 5 hours. A comparison of the bone marrows of ACE and epinephrine injected intact mice indicated that after ACE injection there is an increase in the proportion of eosinophilic to neutrophilic cells while after the epinephrine injection a slight tendency toward a decrease in the ratio of these two types occurs. Since both substances caused an eosinopenia in the circulating blood, the differential effect on the bone marrow can perhaps be interpreted as a direct one of greater stimulation of neutrophile formation by epinephrine.

Summary. Adrenal cortical extract in doses which caused a similar or greater decrease in eosinophiles of intact mice did not produce as extreme a degree of neutrophilia as epinephrine. Even in animals which had been completely adrenalectomized, as indicated by the lack of an eosinopenia after epinephrine, this substance produced a marked increase of neutrophiles in the circulating blood. Neither sex hormones nor peanut oil had this effect. Since epinephrine also caused marked neutrophilia in splenectomized mice its influence was not exerted through the spleen. Preliminary observation of bone marrow smears of intact injected animals indicated a higher proportion of neutrophilic to eosinophilic cells in the marrow of epinephrine injected mice as compared with those injected with ACE. This suggests a direct effect of epinephrine on the bone marrow.

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Intubation of the Pancreatic Duct in the Human.* (18404)

HENRY DOUBILET AND JOHN H. MULHOLLAND.

From the Department of Surgery, New York University College of Medicine and the Third (New York University) Surgical Division, Bellevue Hospital, New York City.

The inaccessibility of the human pancreas and its ducts has restricted direct surgical attack on the diseased gland and thus our knowledge of the physiology of its external secretion is limited. On the other hand, drainage of the biliary tract by means of a T-tube has, incidental to its therapeutic use, increased our knowledge of the variations in hepatic bile secretion. In addition, cholangiographic studies through the T-tube has become an established procedure both in the treatment of biliary tract disease and for studies of the physiologic anatomy of the biliary tract. The few observations on uncontaminated pancreatic juice through the accidental formation of a fistula, have been limited by the difficulty of collecting the juice and by the lack of quantitative studies from the whole gland. Any operative procedure which would enable us to collect human pancreatic juice at will should be of value not only for therapy but also for investigative purposes.

A fortuitious circumstance in a patient with diffuse inflammation of the pancreas in whom drainage of the pancreatic duct was deemed advisable, led to the development of a method herewith described. This procedure is safe and of therapeutic and prognostic value. Furthermore, information concerning the flow and composition of pure human pancreatic juice under various conditions can be obtained.

In the surgical treatment of recurrent pancreatitis by transduodenal section of the sphincter of Oddi, the opening of the duct of Wirung can be found on the posterior wall of the ampulla of Vater which is laid open by this procedure. If not easily discernible the pancreatic duct can be located by the rapid flow of pancreatic juice which is produced by injecting secretin intravenously. A fine polyvinyl tube about 50 cm long is then inserted into the duct for a distance of 8-10 cm. The size of the tube will vary with the diameter of the opening of the pancreatic duct. It should enter freely without occluding the opening. We have been able to insert tubes varying from 1.3 to 1.7 mm in outside diameter.† The other end of the tube is then threaded up the common bile duct and passed through the previously made incision in the bile duct. The end is threaded into the lower arm of an appropriate T-tube and out through a fine opening just above the base of the long arm of the T-tube. The T-tube is inserted into position in the common duct, and the polyvinyl tube pulled carefully until the free loop in the duodenum is fully taken up. Several encircling silk sutures are then tied to hold the polyvinyl tube firmly against the T-tube without undue compression (Fig. 1).

The opening in the anterior wall of the duodenum is closed and the operation completed. Two bottles are attached to the dressing and bile and pancreatic juice are collected separately. The pancreatic juice passes partly through the tube and partly around it into the duodenum. On the fourth postoperative day, the T-tube is tied. The hub of a No. 18 hypodermic needle is broken off, and the blunt end of the needle inserted into the polyvinyl tube. The sharp end is then inserted into the T-tube, so that the pancreatic juice flows down the common bile and into the duodenum.

Samples of pure pancreatic juice, not activated by contact with the duodenal mucosa can be collected at will by pulling the needle out of the T-tube. When a total quantitive collection is required a small dose of morphine (10 mg) is administered subcutane-

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† Transflex tubing no. 20 and 22, Irvington Varnish and Insulator Co.

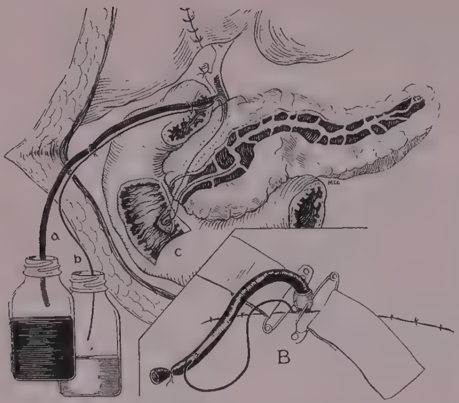

FIG. 1.
The polyvinyl tube (b) passes alongside the T-tube (a), through the lower end of the T lying in the common duct, through the cut sphincter of Oddi (c), and up the pancreatic duct. Bile and pancreatic juice are collected separately. After 4 days the T-tube is tied and the polyvinyl tube inserted into it by means of a needle (inset B), allowing the pancreatic juice to circulate back into the duodenum.

ously. This increases the tonus of the duodenal wall and causes it to contract around the polyvinyl tube lying in the pancreatic duct. This prevents passage of pancreatic juice around the tube into the duodenum and diverts it completely into the collection bottle. A 300 mm length of fine tubing attached to polyvinyl tube produces adequate siphonage to draw all the pancreatic juice from the ducts. The effect of the morphine lasts at least one hour.

Similarly, and most important from a diagnostic and prognostic standpoint, pancreatographic studies can be made by injecting 70% diodrast through the polyvinyl tube. Complete filling of the pancreatic duct system can be attained by giving the patient 10 mg of morphine 5 minutes previous to the injection (Fig. 2).

When the gland appears to be normal both by pancreatographic studies and by analysing the juice for enzymes, the T-tube is removed and along with it the polyvinyl tube. Results of these studies will be reported later.

Summary. A technique is described for intubating the duct of Wirung in the human during the operative procedure of transduodenal sphincterotomy. Through this tube the pancreatic duct can be drained and also visualized roentgenographically. Quantitative studies of the composition of the pancreatic juice under various conditions can be carried out at will.

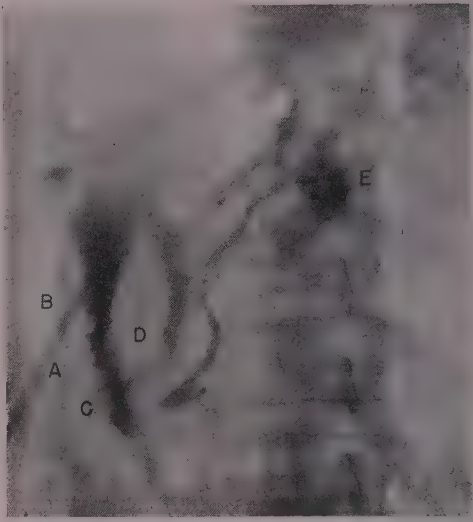

FIG. 2.
Pancreatogram taken one week after operation. Diodrast (70%) was injected into the polyvinyl tube (B), lying alongside the T-tube (A). Its course can be visualized as it curves down the common duct into the duodenum, through the sectioned sphincter of Oddi (C). The pancreatic duct is completely visualized including the accessory duct (D). The tissue of the inflamed tail of the pancreas (E) is rendered opaque by permeation of the Diodrast.

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A Method for the Colorimetric Determination of Calcium in 0.5 ml of Serum. (18405)

ANDRE C. KIBRICK, DORIS PALMER, AND SOL SKUPP.
From the Department of Chemistry, Bronx Hospital, New York

Methods for the determination of calcium which involve titration are not generally convenient for reduced amounts of serum. A colorimetric method which has been widely used on 2 ml of serum consists of an estimation of phosphate after the precipitation of calcium as phosphate(1), but it may be subject to serious error in unskilled hands. Marriot and Howland determined calcium colorimetrically by oxidizing the calcium oxalate precipitate from serum with ferric thiocyanate(2). However, the unmodified method is unsatisfactory since the reagents are not stable and the results are erratic. Although we have succeeded in stabilizing the reagents by using ferric chloride in 18% hydrochloric acid, the results are not quite precise. Rappaport determined calcium by iodometric titration after oxidation of the precipitate of calcium oxalate with excess ceric sulfate(3). Sendroy measured the residual ceric sulfate colorimetrically after addition of iodide(4), and Weybrew, Matrone, and Baxley measured the ceric sulfate directly in the ultraviolet(5). We have found that the excess ceric sulfate can be measured at 420 mu and that results for serum and standard solutions of calcium obtained in this way are apparently more reliable than those determined by the method of Sendroy after addition of iodide. In this paper we describe a method for the colorimetric determination of calcium in 0.5 ml of serum.

Method. Reagents. (1) 4% ammonium oxalate. (2) Dilute ammonia. Dilute 2 ml of concentrated ammonium hydroxide to 100 ml. (3) 1 N Sulfuric acid. Add 27 ml of concentrated sulfuric acid to water and dilute in 1 liter. (4) Cerous sulfate solution. Dissolve 6.25 g of cerous sulfate ($Ce_2(SO_4)_3 \cdot 8\ H_2O$) in 600 ml of water and 56 ml of concentrated sulfuric acid with heat. Cool and dilute to 2 liters. (5) Ceric sulfate stock solution. Dissolve 4.0 g of ceric sulfate, anh., in 500 ml of cerous sulfate by heating to boiling. Cool and filter. Titrate 2 ml of the filtrate with 0.005 N thiosulfate after the addition of potassium iodide, and dilute the balance of the filtrate to 0.0085 N with cerous sulfate solution. (6) Ceric sulfate-cerous sulfate reagent. Dilute 15.0 ml of ceric sulfate stock solution to 100 ml with cerous sulfate solution. (7) Concentrated calcium oxalate standard solution. Dissolve 0.630 g of reagent grade oxalic acid ($H_2C_2O_4 \cdot 2\ H_2O$) and transfer to a 1 liter volumetric flask. Dissolve 0.554 g of reagent grade anhydrons calcium chloride, add 100 ml of 1 N hydrochloric acid, and transfer quantitatively to the flask. Dilute to volume with water. (8) Working standard calcium oxalate solution. Dilute 25 ml of the concentrated standard to 200 ml with 1 N sulfuric acid. 1 ml is equivalent to 10 mg calcium per 100 ml.

Transfer 0.5 ml of serum with Ostwald-Folin pipettes to 5 ml centrifuge tubes and add 0.5 ml of water and 0.25 ml of 4% ammonium oxalate. Mix well with slender rods and allow to stand at room temperature over night. Centrifuge for 10 minutes at 1500-2000 R.P.M. and drain off most of the supernatant fluid (allow about 0.02 ml to remain after drainage) with a fine capillary tube attached to water suction. Wash the precipitate 3 times by adding 0.8 ml of dilute ammonia down the sides of the tubes and stirring the precipitate with a fine glass rod. One rod is used for each tube and rinsed with the succeeding wash solution. During centrifugation the rods are placed in separate test tubes.

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Finally, add 1 ml of 1 N sulfuric acid and return the rods to the centrifuge tubes. Include a blank tube containing 1 ml of sulfuric acid and a standard tube containing 1 ml of working standard with each series of determinations.

Dissolve the precipitate by placing the tubes in a boiling water bath and stirring for 4-5 minutes. Remove the rack of tubes from the bath and allow them to cool for a few minutes. Then add 4.0 ml of ceric sulfate-cerous sulfate reagent, stir, and remove the rods. Finally, mix thoroughly by inversion, using parafilm instead of rubber stoppers, and allow to stand for 15 minutes. Transfer to colorimeter tubes and measure the color in an Evelyn colorimeter with filter 42 at the 6 cc aperture. Obtain the concentration of calcium from the difference in density between the blank and unknown solutions on a standard curve prepared from solutions of known amounts of calcium oxalate. The values obtained for the standard should be close to 10 mg per 100 ml.

In Table I the results obtained by this method in a series of sera are compared with those determined in 2 ml samples by the method of Clark and Collip(7). It is evident that the values agree in every case within 0.3 mg per 100 ml.

TABLE I. Comparison of Results of the New Method for 0.5 ml of Serum with Those Determined by Titration in 2 ml of Serum.

Serum	New method mg per 100 ml	Clark-Collip method mg per 100 ml
1	13.3	13.0
2	13.2	13.4
3	12.4	12.1
4	12.1	11.9
5	10.8	10.9
6	9.7	9.8
7	9.1	9.1
8	9.0	8.9
9	8.8	8.6
10	8.7	8.7
11	8.6	8.6
12	8.5	8.4
13	8.1	8.3
14	7.9	8.0
15	7.6	7.5

Summary. A method is described for the colorimetric determination of calcium in 0.5 ml of serum with ceric sulfate. Data are presented to show that the results agree within 0.3 mg per 100 ml of those determined by titration with permanganate.

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Increased Virus in Eggs Injected with Cortisone. (18406)

EDWIN D. KILBOURNE AND FRANK L. HORSFALL, JR.

From the Hospital of The Rockefeller Institute for Medical Research, New York City.

In the course of investigations of viral infections of the chick embryo, experiments were undertaken to determine the effects of alteration of host physiology upon viral multiplication. Studies by Karnofsky, *et al.*(1), revealing the profound effects of adrenal cortical steroids on the growth and development of the chick embryo, suggested the use of these hormones to alter host metabolism*. Mumps and influenza viruses were employed because of the relative ease and precision with which their concentration may be measured by the hemagglutination reaction.

Materials and methods. Viruses. The PR8 strain of influenza A virus, the Lee strain of influenza B virus, and the Habel strain of mumps virus were employed. 10^4 to 10^5 ID_{50}

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* The generous advice of Dr. David A. Karnofsky, Sloan-Kettering Institute, New York, is gratefully acknowledged.

TABLE I. Comparison of Viral Concentration of Allantoic Fluid Pools from Cortisone-Injected and Control Eggs.

	Control		Cortisone			Cortisone
Virus	Hemagglutination titer*	Infectivity titer	Dose, mg	Hemagglutination titer*	Infectivity titer	Control %
Mumps	158		2.5	744		471
Lee (1)	3810		5.0	10740		282
(2)	1624		2.5	3320		204
(3)	3400	$10^{-6.7}$	1.0	6780	$10^{-8.3}$	200
PR8 (1)	1956		5.0	2406		123
(2)	1866		5.0	2240		120
(3)	814		2.5	1262		155

* As measured by the photometric hemagglutination technic.

was injected into the allantoic sac in 0.1 cc volume.

Cortisone. A saline suspension of cortisone acetate (Merck, 11-dehydro-17-hydroxycorticosterone-21-acetate), containing "suspending agents" and 1.5% benzyl alcohol, was used. 1 to 5 mg in volumes of 0.1 to 0.2 cc was injected into the yolk sac of each egg prior to viral inoculation.

Control material. Benzyl alcohol (1.5%) in 0.85% NaCl solution buffered to pH 7.2 with phosphate was utilized as a control for the cortisone yolk sac injection. (Recent experiments employing the "suspending agents" incorporated in the Merck preparation as a yolk sac injection have disclosed no evidence of increased viral concentration in eggs so injected).

Procedure. Groups of 7 to 10-day old fertile White Leghorn eggs were injected in the yolk sac with cortisone or control material 30 minutes to 48 hours prior to the introduction of virus by the allantoic route. Eggs were incubated thereafter at 35°C for 32 to 52 hours when infected with either influenza virus, and for 131 hours when mumps virus was employed. Embryos were then placed at 4°C for 12 to 18 hours prior to the harvesting of allantoic fluid and embryonic tissues. Embryos were removed, freed from extraneous tissue, and dried with blotting paper before weighing. Hemagglutination titrations were performed with 0.5% suspensions of chicken RBC in the usual manner as well as by a precision photometric technique developed recently in this laboratory. This procedure has a precision of ± 4% in replicate determinations with each of the viruses employed. Infectivity titrations were carried out *in ovo* using groups of 4 to 6 eggs per dilution.

Results. The results of representative experiments are summarized in Table I. The viral concentration of pooled allantoic fluids from cortisone-injected eggs was 120 to 471% of the viral concentration of control eggs as determined by the photometric hemagglutination technic. Allantoic fluids from eggs injected with cortisone but not with virus caused no agglutination of chicken RBC. In all instances the allantoic fluid volume of cortisone-injected eggs exceeded that of controls, militating against fluid concentration as a cause of increased virus in the former. Further evidence of increased viral concentration was provided by the less precise method of comparative infectivity titrations of allantoic fluid pools.

In all experiments the embryo-stunting effect described by Karnofsky(1) was observed; cortisone-injected embryos averaged 32% less in weight than controls. In addition, marked thickening of the chorioallantoic and amniotic membranes and an increase in trichloracetic acid-precipitable material in dialyzed allantoic fluid were noted. This increase in what is presumably protein was demonstrable in allantoic fluids from eggs injected with cortisone alone, and therefore appears to be a phenomenon unrelated to similar protein increases previously described in connection with viral multiplication in the allantoic sac(2).

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Studies of the distribution of virus in cortisone-injected and control eggs are now in progress. Preliminary experiments have disclosed increased viral concentration in embryo tissue from eggs injected with cortisone. It is therefore unlikely that the increased amount of virus in the allantoic fluid is the result merely of a localization or re-distribution of virus in the altered host.

Discussion. The data presented indicate that under the experimental conditions employed the concentration of influenza or mumps viruses attained in developing hens' eggs injected with cortisone may exceed that in similarly infected control eggs by a significant amount. If the concentration of mumps virus in cortisone-injected eggs is multiplied by a factor expressing the increased allantoic fluid volume of such eggs, it is found that the increased yield of virus per egg may be as high as 688% of controls.

Coincident with the observed increases in the amount of virus formed, profound changes in the host occur which are attributable to the effects of cortisone(1). Because the mechanism of cortisone effects upon the host is obscure, speculation concerning the basis for increased viral concentration is not warranted at present. It should be recalled, however, that the chick embryo is not known to possess immunologic mechanisms so that the suppression of antibody formation observed with cortisone(3) cannot be invoked in explanation of the present phenomenon.

Recent studies by Kalter, *et al.*(4) on the effect of growth hormone and testosterone on influenza virus multiplication in mice revealed increases in the "rate of virus proliferation and . . . the amount of virus present" in lungs of hormone-injected animals. These increases were related by the authors to increased host protein anabolism induced by hormone administration. In contrast, cortisone injection depresses chick embryo growth and may produce weight loss and negative nitrogen balance in the rat(5), but nonetheless results in increased viral concentration in the infected host in which anabolic function is apparently depressed. These findings constitute a paradox only if the dual assumption is made that: (1) viral concentration is directly related to host protein anabolism, and (2) cortisone actually causes depressed anabolism of the developing egg. Present evidence is insufficient to justify either assumption.

Summary. The concentration of Lee, PR8, or mumps virus in the allantoic fluid of eggs injected with cortisone acetate is significantly greater than the concentration of these agents in the allantoic fluid of control eggs.

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Non-effect of a Growth Hormone Preparation in the Regenerating Feather.* (18407)

MARY JUHN.

From the Department of Poultry Husbandry, University of Maryland, College Park.

An acceleration of feather growth normally occurs in the fowl during the juvenile molts when the bird is in the phase of rapid development. Such accelerations occur in association with the appearance of new pigment—and structural traits, as may be seen when the measured axial increments of successively regenerating feathers from given follicles are compared in relation to the respective pat-

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* Scientific Paper No. A289 Contribution No. 2236 of the Maryland Agricultural Experiment Station (Department of Poultry Husbandry).

terns displayed(1). The converse situation, a slowing down of feather growth is obtained by feeding the anti-thyroid drug thiouracil. In hybrid fowl this experimental retardation has as a clear sequel an alteration of the plumage phenotype, bringing further evidence of participation by the growth properties of the system in the development of its pattern(2).

There are certain indications which suggest that the action of estrogens in determining female feathering may proceed *via* a primary alteration imposed upon the zone of growth of the feather germ by the hormone (3). This supposition could be verified were it possible to approximate regional feather growth in the male of a sexually dimorphic breed to that of the female by some substance free of estrogenic properties and having no intrinsic action on feather structure or color. Growth hormone preparations could be expected to yield accelerated feather growth and seemed entirely suitable on such other grounds. Admirable test fowl are Brown Leghorn males, where the dorsal feathers grow more slowly, the ventral feathers more rapidly, than do the corresponding ones of the hen. Induced growth accelerations of the male feather hence might cause simultaneous female patterns. Similar morphological alterations should however, not accompany artificially increased rates of growth in the male breast plumage.

Experimental methods. The growth hormone preparation (Lot 22KRI) used was most kindly furnished by Dr. I. M. Bunding, Armour and Company, Chicago, Illinois. It was stated to cause an increase of 20 gram body weight in 15 days in the hypophysectomized rat; the daily dose being 50 gamma per 50 gram body weight. Portions of this powder were dissolved in 0.9 per cent saline in distilled water, 10 or 20 gamma per ml. Amounts sufficient for one day's treatment were stored frozen in vials and thawed only just prior to intradermal injection. The birds treated with the growth hormone preparation were adult Brown Leghorns, three cocks, one slip, and two capons weighing 1548, 1570, 1905, 2025, 2130 and 2323 grams, respectively. The times of emergence of new feather germs and the later feather growth rates normal to each of the 6 separate birds were first established as a basis for comparison. Feathers were plucked May 25, 1949, on the right side of the body and the birds were examined at intervals thereafter for the first appearance of the feather germs beyond the mouth of the follicle. Records were then made thrice weekly of the axial increments of eight regenerates in the saddle and nine in the breast of each fowl. The original feathers of these rows were filed individually for later pattern reference. Plumage patches at some little distance were denuded simultaneously to provide regenerating material for split feather germs. These preparations permit an inspection at any desired interval of currently induced modifications(4).

June 15, 1949, this same procedure was repeated at identical levels of the body but on the left side. Commencing June 25, to July 8, incl., each bird received 40 gamma of the growth hormone daily. 20 gamma were given in the morning and 20 in the evening. Half of this dosage was injected very slowly each time immediately anterior of the regenerating row of feathers in the left saddle and of the row in the left breast. This method of administration was retained but the daily amounts injected were increased to 160 gamma daily from July 9, to July 18, and to 320 gamma daily from July 19, to July 27. A second plucking in the left saddle and breast was made immediately forward of the sites of injection of the growth hormone July 8, 1949.

Results and discussion. The breast feathers grown in the test periods deviated in no manner from the normal; they are omitted from further discussion.

The saddle feathers plucked May 25 had laid down about the first third to one-half of their length at the time the injections of the growth hormone were commenced on the

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opposite side of the body. The feathers of the left saddle plucked June 15 were emerging beyond the mouth of the follicle at this time and they achieved about half their growth during treatment. The regenerates of the July 8 plucking completed all the phases of reconstitution and their first growth took place in the presence of the growth preparation.

Comparisons of the course of regeneration under these three separate conditions were made between derivatives of follicles of substantially identical position in the body. There proved to be no detectable effect on the part of the growth hormone (in the concentrations employed) either in acceleration of axial growth of the feather or in reduction of the time occupied by the restitutory processes. Serial split preparations showed, however, that during injection of the growth hormone a stimulation of thyroid activity occurred. Black pigment granules were found deposited in the zone of growth; this is an effect characteristic of excess thyroxine in the Brown Leghorn(5). Tests for traces of thyrotrophin in the growth preparation were therefore made following the method of Smelser(6).

Four groups of ten each male and female New Hampshire chicks, four weeks of age, were selected from the University of Maryland stock for a similar distribution by body weight. Ten, 40, and 160 gamma respectively were given daily to one group each, the dose divided between morning and evening. The fourth untreated group served as control. Half the number was sacrificed the day after completion of five days of treatment; the remainder upon the eleventh day after 10 days of injection. The thyroids were dissected free of surrounding tissue and weighed on a Roller-Smith balance. Treated and control glands were not found to differ in either series. However, Smelser has pointed out(5) that the thyroid of the 6-day-old thyrotrophin treated chick may show signs of activity at dosages less than those causing a weight increase. The saddle feather germ of the Brown Leghorn accordingly seems a more sensitive indicator than thyroid weight.

Early negative results often are later found due to inadequate concentrations. Consequently the daily dosage of the growth hormone was increased about 30-fold. One Brown Leghorn cock weighing 2005 grams was given 10 mg daily for 5 successive days. The effect on feather growth was again negative but the thyroid was more markedly stimulated as judged by the intensified reaction recorded in the feather germ. Failure to obtain growth acceleration could still result from dosage insufficiency but it also may be that the traces of thyrotrophin in the preparation interfered in some manner with a possible response.

Summary. An attempt was made to cause acceleration of feather growth in fowl by injections of growth hormone in the vicinity of regenerates. The preparation used gave a 20-gram body weight increase in 15 days in the hypophysectomized rat in dosage of 50 gamma per 50 grams body weight. Six Brown Leghorn males each received 40, 160, and 320 gamma over a period of weeks. One fowl was given 10 mg daily for 5 days. All tests were negative in feather growth but induced pattern changes indicated the presence of traces of thyrotrophin in the preparation.

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Role of Carotid Chemoreceptors in Hypoxic Cardiac Acceleration (18408)

THEODORE BERNTHAL, WILSON GREENE, JR., AND ALVIN M. REVZIN.
From the Department of Physiology, Medical College of the State of South Carolina, Charleston, S. C.

Though the increased heart rate which accompanies acute hypoxia is well known, the mechanism of its genesis is incompletely understood. Sands and DeGraff(1) and Wiggers(2) have shown that it depends chiefly upon reduced tonic activity of the vagal cardio-inhibitory center and in lesser degree upon increased activity of the sympathetic cardio-accelerator center. However, the channels through which hypoxia operates when it alters the action of these centers have not been demonstrated.

The suggestion has been made repeatedly (3-8) that the changes in heart rate which accompany alterations of arterial oxygen tension are initiated at the chemoreceptors of the carotid and aortic bodies. Thus, it has been assumed either that hypoxic stimulation of these chemoreceptors would reflexly accelerate the heart or, conversely, that hyperoxic diminution of their tonic activity would do the reverse. Since the literature seemed devoid of experimental evidence bearing directly upon these assumptions, we have performed experiments to determine the chemoreflex response of heart rate to hypoxia. The results do not support the suggestion that hypoxic cardiac acceleration is initiated at the carotid or aortic bodies.

Method. Our procedure consisted simply of recording heart rate during the application of hypoxic stimuli carefully restricted to the carotid chemoreceptor region. Dogs were anesthetized with morphine sulphate (4 mg/kilo) and urethane (1 g/kilo) and their heart rates recorded from a carotid artery with a membrane manometer. Breathing was recorded to indicate the intensity of chemoreceptor stimulation. While preserving Hering's nerves, all vessels efferent to each carotid bifurcation were ligated a few millimeters beyond their origin. The vascularly isolated carotid regions were then supplied with blood from a perfusion source, the blood entering through cannulae placed in the common carotid arteries and leaving through cannulae in the external carotid arteries from which it returned to the perfusion source through an adjustable resistance. During controlled periods, normally aerated heparinized blood flowing in this circuit was rendered hypoxic in known degree. In some instances, the perfusion source was a donor animal inhaling a gas mixture low in partial pressure of oxygen for short periods. In other experiments, autoperfusion apparatus was used(9). Cyanide was administered by injecting directly into the carotid perfusion circuit. In most experiments, arterial blood pressure compensators(9) minimized variations of pressoreceptor stimulation. In some experiments, natural breathing was allowed. In others, constant artificial pulmonary ventilation with open pneumothorax was imposed.

Results and discussion. Representative responses of eight animals are summarized in Table I which gives only the maximum deviation of heart rate from control values during each hypoxic episode. In Fig. 1, plotted summaries of the heart rate during 3 complete hypoxic episodes and one cyanide administration are given in order to show the pattern and time relationships of the responses. Here

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TABLE I. Effects of Hypoxic Stimulation of Isolated Carotid Chemoreceptors Upon Heart Rates of Dogs.

		Heart rate—beats/min.							
		Natural pulmonary ventilation				Controlled (constant) pulmonary ventilation			
Exp. No.	Stimulus*	Before	During†	After	% change	Before	During†	After	% change
1	1% O_2	186	180	186	— 3.2				
2	7% O_2	201	204	201	+ 1.5				
3	1% O_2	204	204	204	0.0	189	174	183	— 7.9
4	4% O_2	191	155	191	—18.3	216	141	217	—34.7
5	4% O_2	95	103	90	+ 8.4	(Donor: +17% during same procedure)			
	8% O_2	89	93	93	+ 4.5	(Donor: +8.4% during same procedure)			
6	4% O_2	198	211	194	+ 6.5	248	240	251	— 3.2
	2% O_2					181	172	180	— 4.9
	NaCN, 1 cc	260	284	264	+ 9.0	252	246	260	— 2.3
	NaCN, 2 cc					244	215	248	—11.0
7	4% O_2	150 ± 3	150 ± 4		0.0	209 ± 3	184	202	—12.0
	4% O_2					201	182	193	— 9.4
	Whole animal—constant ventilation: 8% O_2: 175/min. to 224/min. = +29%.								
	NaCN, 0.5 cc	112	124	116	+11.0				
8	4% O_2	135	136.5	135	+ 1.0				
	2% O_2	135	142	136	+ 5.0	201	202	200	+ 0.5
	NaCN, 1 cc	148	112	148	—24.0	191	113	189	—40.8
	Whole animal—natural breathing: 8% O_2: 150/min. to 220/min. = +46% 4% O_2: 130/min. to 213/min. = +63%								

* O_2 values = pO_2 in % of one atmosphere in inspired air of donor supplying blood to carotid perfusion circuit.

NaCN values = amount of 0.01 N solution injected into carotid perfusion circuit.

† Values in columns 4 and 8 pertain to the 30 sec. interval showing maximum deviation from control.

it can be seen that, although the changes were small, they coincided definitely with the period of carotid hypoxia, were well maintained during that time, and were reversed when the hypoxia was ended.

As Table I shows, the reactions were qualitatively consistent only when pneumothorax and constant artificial ventilation of the lungs were maintained. Under these conditions the heart rate was diminished in all instances of hypoxemia and cyanidemia at the chemoreceptors except 2 in which there were no significant changes and *in no instance was there cardiac acceleration.* On the other hand, when natural breathing was allowed so that pulmonary ventilation and stretching of the lungs were increased during chemoreceptor stimulation, the heart rate diminished in some instances, was unchanged in others and increased moderately in the remainder. Of the two sets of reactions, the first is taken to be the more nearly representative of direct uncomplicated chemoreflex responses since variable factors secondary to the reflex hyperpnea which results from chemoreceptor stimulation had been eliminated or lessened by controlling the breathing. The finding that the direct reflex effect of hypoxemia at the carotid bodies is slowing rather than acceleration of the heart harmonizes with the similar finding for cyanide stimulation (Exp. 6 and 8, Table I, and Fig. 1). Reflex bradycardia due to cyanide is well known(10) and the response to lowered oxygen tension might have been anticipated upon this basis since the effects of chemoreceptor stimulation could be expected to remain qualitatively the same whatever the identity of the stimulating agent.

In the experiments with natural ventilation, there were factors presumably secondary to the increased breathing which tended to cause cardiac acceleration and when sufficiently forceful they counterbalanced or overbalanced the direct reflex effect. Thus, in Experiment 6, a relatively moderate chemoreflex slowing was overbalanced by the secondary accelerating influence of the increased breathing, with a net effect of cardiac acceleration. In Experiment 3, a stronger reflex slowing apparently

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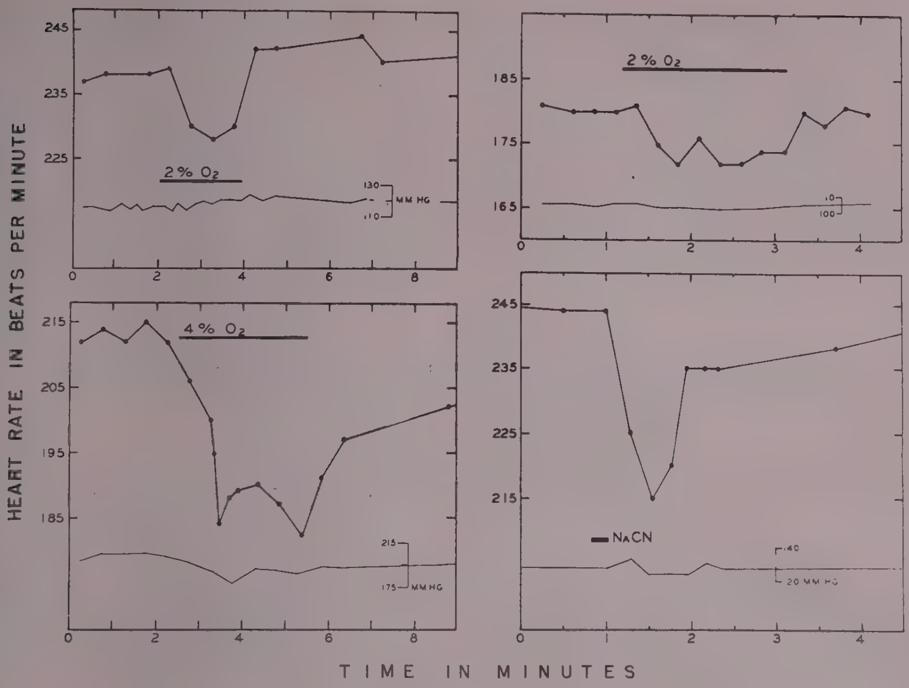

FIG. 1.

Changes in heart rate of dog during hypoxemia and cyanidemia restricted to the carotid bodies.

Percentage values: partial pressure of oxygen, in percent of one atmosphere, in inspired air of donor supplying arterial blood to isolated carotid bifurcations of experimental animal. Black bar: period during which low oxygen mixture was inhaled.

NaCN dosage: 2 cc of 0.01 N solution injected into carotid perfusion circuit.

Lower thin line: mean arterial blood pressure of experimental animal.

just counterbalanced the secondary effect so that no change of heart rate occurred. In Experiment 4, very marked chemoreflex slowing completely overbalanced the secondary acceleratory influence so that slowing occurred during both natural and artificial breathing, although less markedly in the former.

The nature of the cardiac acceleratory factor which is secondary to increased breathing and which disappears during constant ventilation with open chest is not evident although reflex inhibition of the cardio-inhibitory center resulting from increased inspiratory stretching of the lungs as analyzed by Anrep, Pascual and Rössler(11) would seem to be a leading possibility. Whatever its nature, it appears unlikely that this factor is an essential part of the mechanism producing cardiac acceleration when the whole animal is subjected to hypoxia. The cardiac acceleration of generalized hypoxia usually is not reversed by the use of constant pulmonary ventilation (see Exp. 7, Table I). Further, in none of these experiments was the acceleration secondary to chemoreflex hyperpnea as much as 10% of control values, whereas generalized hypoxia produced increases of 30 to 60% or more (Exp. 7 and 8, Table I).

We conclude, therefore, that the cardiac acceleration of generalized hypoxia does not depend upon the carotid chemoreceptors, although effects secondary to chemoreflex hyperpnea may contribute in minor degree. It might be added that the same probably holds true for the aortic chemoreceptors, since all known aspects of their function are quali-

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11. Anrep, G. V., Pascual, W., and Rossler, R., *Proc. Roy. Soc. London,* 1935-36, v119B, 191, 218.

tatively like those of the carotid bodies. Moreover, Comroe(12) has demonstrated that their stimulation by cyanide or lobeline produces a reflex bradycardia just as does that of the carotid chemoreceptors.

Summary and conclusions. 1. Changes in heart rate were recorded during the application of hypoxic stimuli limited to the carotid chemoreceptors. 2. The direct reflex change in heart rate initiated by hypoxia at the carotid chemoreceptors was a slowing of moderate degree. 3. It is concluded that the cardiac acceleration of generalized hypoxia does not arise chemoreflexly at the carotid bodies.

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Further Observations on Yeast in Production of Experimental Hepatic Necrosis in Rats.* (18409)

PAUL GYÖRGY AND HARRY GOLDBLATT.

From the Nutritional Service, School of Medicine, University of Pennsylvania, Philadelphia, and The Institute for Medical Research, Cedars of Lebanon Hospital, Los Angeles.

It has been shown that in rats fed a diet deficient in tocopherol, with yeast as the sole source of protein, the occurrence of massive hepatic necrosis may vary considerably in direct relation to the brand of yeast used(1). Incorporation of a British brand of baker's yeast in the diet led to massive necrosis with great regularity (90-100%). In contrast, substitution of our American brand of brewer's yeast in the same ration deprived it of its "necrogenic" quality. By increasing the fat-content (lard) in the mixture up to 50%, necrosis occurred, even with the American brand of yeast, but the incidence remained relatively low around 50%(2). The necrogenic activity of the British yeast could not be explained by a difference in the content of the sulfur-containing amino acids (cystine, methionine) or of vit. E in these particular British and American brands of yeast. The fact that the British yeast used was a live baker's yeast and the American yeast a heat-killed brewer's yeast made it desirable to test (1) an American live baker's yeast for its necrogenic activity, and (2) to determine whether the British yeast retained its necrogenic character after inactivation through autoclaving.

Experimental. As in our previous experiments(1,3) the following "necrogenic" experimental diet was used: yeast, 18; corn starch, 79; salt mixture (U.S.P. II), 3%. Peanut oil, 0.5 ml and cod liver oil, 2 drops were added to 8 g of the dry mixture just before feeding. Further, all animals received daily supplements of 20 γ of thiamine, 25 γ of riboflavin, 20 γ of pyridoxine, and 100 γ of calcium pantothenate dissolved together in 1 ml of water, as well as 20 γ of vit. K. We used the British† (in diet Y5H) and an American‡ (in diet Y5F) type of baker's yeast.

In Exp. II, the necrogenic activity of the live British yeast was compared with that of the same brand of yeast after being autoclaved (for 5 minutes with free flowing steam, followed by 25 minutes steam sterilization at 17 lb per square inch, then a 14-inch vacuum

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* Supported by a grant of Standard Brands, New York and a grant (to H. G.) from the Division of Research Grants and Fellowships of The National Institute of Health, U. S. Public Health Service.

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† Obtained under the designation "D.C.L. Vit. B_1 Yeast" from Distiller's Co., Glasgow.

‡ Type 753-powdered Fleischman's baker's yeast. Furnished by Standard Brands, New York.

is drawn for 40 minutes).§ Male rats of the Sprague-Dawley strain were used. The weight of the rats at the start of the experiments varied from 45.0-70.0 g. All rats were kept in single cages, with raised, wide meshed screen bottoms. The daily food intake was measured.

TABLE I. Necrogenic Effect of British and American Baker's Yeast.

Exp. I	Diet Y5H with British yeast	Diet Y5F with American baker's yeast
No. of rats	12	13
Avg initial wt (g)	57.0 ± 0.7	56.5 ± 0.8
Food intake (avg) g/day	6.5 ± 0.2	6.9 ± 0.2
Avg survival time (days)	51.4 ± 3.5	64.0 ± 5.3
Massive necrosis at death (rats)	12	12*

Exp. II	Diet Y5H	Diet Y5H autoclaved
No. of rats	13	14
Avg initial wt (g)	48.5 ± 0.6	49 ± 0.55
Food intake (avg) g/day	5.9 ± 0.1	5.6 ± 0.1
Avg survival time (days)	38.9 ± 1.7	36.6 ± 2.4
Massive necrosis at death (rats)	13	14

* One additional rat died after 32 days, without signs of necrosis.

The results as summarized in Table I, illustrated by Exp. I, indicate that the American baker's yeast, in contrast to the previously used(1) American brewer's yeast, was just as conducive to the production of massive hepatic necrosis as the British baker's yeast. The average survival time of the experimental rats was greater when the ration contained American baker's yeast, than with the British brand of baker's yeast. A statistically significant difference in survival time was obtained in another experiment in which rats with slightly higher average initial weight (66.5 g) were used. Here the survival time was prolonged in both groups, receiving the British and American brand of yeast, probably due (4) to the higher initial vit. E reserves of the rats used. The average survival time of the rats receiving the British brand of yeast was 98.5 days (± 4.5), that of the rats fed the same basal diet containing the American brand of yeast, 153.6 days (± 6.3). Thus, it may be postulated that in general the American baker's yeast, although sufficiently necrogenic, is slightly less potent than the British baker's yeast. This difference appears to be related to a better protection of the store of vit. E in the living animal by the American brand of baker's yeast. The necrogenic activity of an American brand of baker's yeast has been demonstrated recently also by Abell, Beveridge and Fisher(5).

In contradistinction to the heat-killed, non-necrogenic American brewer's yeast(1), the necrogenic British and American baker's yeasts are live yeasts. The possibility that such active yeast may be less well digested and utilized(6), could have a bearing on its necrogenic potency. In Exp. II (Table I) comparison was made between active and autoclaved British yeast of the same original batch. The results obtained show unequivocally that heat-killed yeast was just as necrogenic as the same yeast before heat-inactivation.

Conclusions. An American brand of baker's yeast was found to be only slightly less necrogenic than the previously used British baker's yeast. The necrogenic activity of the British yeast was not diminished by heat-treatment (autoclaving).

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§ This experiment was originally devised as preliminary approach to studies on the effect of the necrogenic yeast diet in germ-free animals, to be conducted at the Germ-Free Life Division of Lobund, University of Notre Dame. The preparation of both, autoclaved and non-autoclaved food mixtures used in Exp. II was carried out under the supervision of Dr. T. D. Luckey at Lobund, U. Notre Dame.

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Influence of Digilanid on Creatine Reserve of Normal, Thyrotoxic and Hypothyroid Rat Ventricle.* (18410)

Albert Sjoerdsma.† (Introduced by E. M. K. Geiling.)

From the Department of Pharmacology, The University of Chicago.

Only a relatively few reports have appeared concerning the action of digitalis substances on the metabolism of hearts in failure. Since doses of digitalis which are toxic to normal hearts may actually improve the competency of insufficient hearts, it is likely that the chemical changes induced are different in each case. Clinically, it is not unusual to find heart failure in the presence of hyperthyroidism and hypothyroidism. Thyroxin injections and thyroparathyroidectomy offer easy means whereby these clinical states may be simulated in rats.

Several investigators(1-7) have established the fact that low cardiac creatine levels usually accompany myocardial failure in humans. Beltrametti(8) showed that during heart failure there is a marked creatinuria which gradually disappears as circulatory function is restored toward normal. Experimentally, Cowan(9), Bodansky(10) and Bodansky and Pilcher(11) succeeded in effecting a loss of creatine from rat heart by producing hyperthyroidism in their animals. Cowan(9) also reported that thyroparathyroidectomy produced a small but significant decrease in the creatine content of rat heart. Decherd, Herrman *et al.*(12,13) measured the effects of cardiac glycosides on the creatine reserve of hypertrophied rabbit hearts with sectioned aortic cusps. They found that digitalization alone had a tendency to increase the creatine content and seemed to offset the decrease in creatine percentage of great hypertrophy. These findings indicated that it might be of interest to measure the creatine reserves following the administration of Digilanid to rats in which an altered thyroid state had been induced.

Methods. The animals used were Sprague-Dawley rats ranging in weight from 230 to 300 g. For injection, synthetic thyroxin (Hoffman LaRoche) was dissolved to a concentration of 1 mg per cc in water made slightly alkaline with sodium hydroxide. The Digilanid solution contained 0.2 mg of Digilanid per cc, plus 7.5% alcohol and 15% glycerine by weight. Groups of 4 to 6 animals were injected intraperitoneally with one or both drugs given daily in 1 cc doses. Thyrotoxicosis was produced in all cases by injection of thyroxin for 21 days. Two sets of experiments were carried out on thyrotoxic animals. In the first set Digilanid was administered for 10 days to rats which had been pre-treated with thyroxin for 11 days. In the second set Digilanid was given for a total of 28 days, the animals to be thyroxinized being pretreated with Digilanid for 7 days. Thyro-

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† Public Health Service Research Fellow of the National Heart Institute.

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TABLE I. Creatine Reserve of Digitalized Normal. Hyperthyroid and Hypothyroid Rat Ventricle.

Exp.	Treatment[*]	No. of animals	Creatine, mg %	T[‖] values	Initial[†] vent. wt, mg	Final[‡] vent. wt, mg	Stand. dev.	VW/BW[§] final	Initial[†] creatine content, mg	Final[‡] creatine content, mg
I	Normal	13	188	—	653	890	35	2.61	1.23	1.67
	D-10	6	155	9.7	—	—	—	—	—	—
	T-21	4	114	17.5	—	—	—	—	—	—
	D-10, T-21	4	114	12.9	—	—	—	—	—	—
II	D-28	5	203	2.2	630	940	30	2.79	1.18	1.91
	T-21	5	136	16.2	642	1100	40	4.20	1.21	1.50
	D-28, T-21	5	133	10.3	631	1000	56	4.21	1.19	1.33
III	Normal	6	190	—	689	746	13	2.79	1.31	1.42
	D-28	4	191	0.08	714	759	49	2.83	1.36	1.45
	TP-21	4	213	3.04	717	650	15	2.34	1.36	1.52
	D-28, TP-21	5	222	8.88	714	578	21	2.21	1.36	1.28

[*] D, T, and TP refer to Digilanid (0.2 mg/day), Thyroxin (1 mg/day), and Thyroparathyroidectomy, respectively. Numerals represent number of days induced state was continued before animals were sacrificed.

[†] Calculated by use of the normal VW/BW.

[‡] Observed values.

[§] VW/BW equals mg of ventricular wt per g body wt.

[‖] Significance of differences between means; less than 3 is not considered statistically significant(15).

$$T = \frac{M_1 - M_2}{\sqrt{E_1^2 - E_2^2}} \qquad (E = \text{mean error}, M = \text{mean}).$$

parathyroidectomies were done on normal rats and rats pre-treated with Digilanid for 7 days. In the latter case, Digilanid injections were continued for an additional 21 days before the animals were sacrificed. Analyses were always carried out 21 days post-operatively. Litter mates served as controls. The animals were fasted for 24 hours before being sacrificed by decapitation. The thorax was opened at once and the ventricles were precisely separated from the auricles, divided into 2 approximately equal portions, blotted dry and weighed on a torsion balance. No attempt was made to distinguish right from left ventricle. One sample was used for creatine determination, the other for dry weight measurement or other analyses. The method of Rose, Helmer and Chanutin(14) as modified by Myers(7) was used for the estimation of total creatine in rat ventricle.

Results. The results of the creatine determinations are given in Table I. The normal controls for experimental Groups I and II showed an average creatine concentration of 188 mg %. In Exp. I it will be noted that injections of thyroxin (1 mg/day for 21 days) and Digilanid (0.2 mg/day for 10 days) effected a decrease in the creatine concentration of rat ventricle. Thyroxin produced the greatest decrease, amounting to about 39% below the normal value. Treatment of thyroxinized animals with Digilanid for 10 days had no effect whatsoever on the already lowered creatine percentage.

In Exp. II Digilanid was administered for 28 days to ascertain whether a longer period of digitalization would alter the percentage decrease of creatine in the hyperthyroid rat heart. The animals were weighed at the beginning and end of the experimental period in order to estimate changes in ventricular weight and total creatine content during the experimental period. Digilanid alone seemed to raise the creatine percentage slightly but this difference was found not to be statistically significant (T value less than 3.0). Other findings were essentially the same as in the previous experiment; thyroxin caused a drop of about 28% and this change was not affected by Digilanid. The theoretical average initial ventricular weight was calculated by multiplying the initial body weight by the ventricular weight/body weight ratio of normals. It was then possible to calculate the

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theoretical initial creatine content of each heart, using the normal value of 188 mg % as the creatine concentration. Final ventricular weight was measured directly and final content of creatine could be calculated from the creatine percentage as determined. Final V.W./B.W. ratios were measured directly. The animals receiving thyroxin lost weight during the experimental period and exhibited cardiac hypertrophy which was apparent grossly and in the final V.W./B.W. ratio, which was 4.20 as compared to the normal of 2.61. Digilanid decreased this hypertrophy somewhat but a final ratio of 4.21 showed that there was no relative change. The actual increase in total creatine content of thyroxinized ventricle during the experimental period was about 65% of the normal increase, and in the case of animals receiving Digilanid as well, only 35%. Thus, the effect of thyroxin in causing a relative loss of creatine from the hearts was increased by Digilanid. In spite of the diminished concentration of creatine in hyperthyroid ventricle the cardiac hypertrophy more than compensated for the difference to keep the total amount from decreasing.

In Exp. III similar studies were done with thyroparathyroidectomized rats. Again, Digilanid for 28 days did not significantly alter the creatine percentage. Thyroparathyroidectomy was followed by an increase of questionable significance, while the operated animals which were given Digilanid for 28 days showed a definite increase in creatine concentration. There was a clear cut loss of ventricular mass in those animals which were thyroparathyroidectomized, and this cardiac atrophy was increased by Digilanid. These changes are reflected in the lowered V.W./B.W. ratios, 2.34 and 2.21 as compared to the normal of 2.79. Digilanid produced an actual loss of creatine in operated animals but had no detectable effect on normal animals in this regard.

Measurements of dry weight of ventricles from drug-treated animals agreed closely with the normal values. Thus, where atrophy or hypertrophy occurred it was real and not due to changes in water content.

Discussion. The purpose of these experiments was to measure the action of Digilanid on the creatine reserve of normal rat hearts and to compare these results with any effects which might be produced in animals with an altered thyroid state. Digilanid administered to normal rats for 10 days effected a decrease in creatine concentration of about 18%. Following 28 days of Digilanid treatment, the creatine concentration, ventricular weight and total creatine content were found to be within normal limits. Digilanid had no influence on the percentage decrease in creatine produced by thyroxin injections, but did diminish the hypertrophy of the thyrotoxic ventricle and as a consequence increased the relative loss of total creatine. Although thyroparathyroidectomy elevated the creatine concentration slightly, concomitant cardiac atrophy accounted for an essentially normal total creatine content. The only statistically significant effect of Digilanid in the operated animals was to enhance the cardiac atrophy, thus leading to an actual loss of creatine from the hearts.

It is apparent then that Digilanid did not change appreciably the cardiac creatine concentration of normal, thyroxinized and thyroparathyroidectomized rats. Alterations in total creatine content were a direct consequence of diminished heart muscle mass. It is not known whether the cardiodynamic state of the animals used was affected by Digilanid but presumably the changes in heart size indicate that such was the case. Nonetheless, no correlation of such effects with the creatine concentration of the heart muscle can be made at this time.

Summary. Under the conditions of these experiments Digilanid (0.2 mg per day for 28 days) exerted no direct effect on the concentration of creatine in rat ventricle. However, in the animals with altered thyroid states, Digilanid brought about an actual decrease in ventricular mass which was reflected in a relative or absolute fall in the total creatine content.

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Fertilization of Undenuded Rat Ova.* (18411)

ROGER H. BOWMAN. (Introduced by S. L. Leonard.)
From the Department of Zoology, Cornell University, Ithaca, N. Y.

It was recently shown that rat ova can be fertilized preceding denudation of their follicle cells(1,2). Since the precise location of fertilization was not reported, it was desired to know if ova, surrounded by follicle cells and located in the oviduct, were still capable of being fertilized. Reports of previous investigators, based on an estimated time of ovulation, suggest that fertilization may occur in the oviduct(3-5), by none has shown that ova were actually in this part of the tract and surrounded by follicle cells at the time fertilization occurred. The present experiment will show that such ova can be fertilized.

Methods. It has been indicated that both ovaries of the mouse probably ovulate within a short time of one another(6,7); the same holds true for the rat(8). With these observations as a basis, the experimental procedure was to (a) breed rats late in heat, (b) immediately remove and examine one oviduct for the presence of ova, and (c) after a suitable period of time recover ova from the other oviduct and determine if fertilization had occurred. Rats which were in heat in the evening, as observed by lordosis when tested with males, usually remained in heat until at least 8 o'clock the following morning. Since the rat ovulates during late estrus(9) the animals in this experiment were bred between 5 and 7 o'clock of the morning following observed evening heat. Within a few minutes after ejaculation the oviduct and ovary were removed from one side. Those animals in whose oviducts no ova could be found were discarded. Four to 9 hours after copulation the animals were killed, and the remaining oviduct removed. The loop containing the ova was then transferred to a microscope slide and the ova pressed out into a saline solution. They were examined for the disposition of follicle cells, which, if present, were removed by hyaluronidase(10). The occurrence of fertilization was then determined by the presence of spermatozoa within the ova.

Results. Nineteen animals had ovulated at the time of copulation, and 98 ova were obtained from the remaining oviducts of these animals. Table I shows that 34 of the ova were fertilized, and that all but 2 of these were enveloped by follicle cells when recovered.

TABLE I. Fertilization of Rat Ova.

Animal No.	Bred before sunrise*	Hr between copulation and autopsy	Follicle cells surrounding ova	Ova recovered	
				Fertile	Non-fertile
1	+	7	+	2	3
2	+	7	+	4	1
3	+	7	—	0	4
4	+	7	+	9	1
5	+	7	—	2	3
6	+	7	+	2	0
7	+	7	+	4	0
8	+	7	+	0	6
9	+	9	+	1	5
10	—	6	+	5	1
11	—	6	—	0	5
12	—	9	—	0	6
13	—	5	+	0	3
14	—	5	—	0	3
15	—	5	+	0	7
16	—	5	+	0	4
17	—	5	+	0	6
18	—	4	+	5	0
19	—	5	+	0	6

* Period of experiment: Feb. 24 to May 6, 1950.

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* Aided by a grant from the Sage Research Fund of Cornell University.

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Lewis and Wright(7) expressed the view that the mouse ovum is always fertilized before loss of its follicle cells, and Leonard, Perlman, and Kurzrok(1), as well as Austin(2) demonstrated that the recently fertilized rat ovum is usually found still enclosed by these cells.

In these previous experiments, however, no attempt was made to determine where fertilization occurred. There exists the possibility that as the ovum is forced from the ovary the associated follicle cells might be pulled aside sufficiently to facilitate entrance of the awaiting sperm. A subsequent closing-in of these cells would then give the impression that the sperm had worked its way through the entire surrounding follicle cell mass. In the present experiment, however, it was known that the ova surrounded by their follicle cells were in the ampulla of the oviduct before arrival of the spermatozoa, and yet the sperm were able to effect fertilization.

It will be noted in Table I that the first 9 animals yielded 24 fertilized ova out of a possible 47 recovered, whereas of the remaining animals, only 2 possessed ova with sperm. These first 9 animals were all bred before sunrise, but due to progression of the spring season the remaining rats were bred after sunrise. It is possible that with the approach of longer days, the ovulation time was shifted to an earlier period with the result that the inseminations may have taken place too long after ovulation for fertilization to succeed. It is true that the lapsed time between insemination and examination of the ova for sperm was somewhat shorter in the animals bred after sunrise, but one rat had ova fertilized by 4 hours post-coitus. It could not be for the lack of sperm that fertilization failed in these animals since spermatozoa were observed in all of the oviducts but one. Blandau and Money(11) have reported spermatozoa in the ovarian portion of 100% of rat oviducts examined one hour after copulation.

Summary. Rats were bred late in estrus and the presence of ova in the oviducts determined by immediate removal and examination of one of the ducts. By later removal and examination of ova from the other oviduct, it was possible to demonstrate that rat sperm could fertilize non-denuded ova within the oviduct. The incidence of fertilization was markedly lowered by copulation late in estrus.

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Determination of Urea in Saliva. (18412)

DAISY YEN WU AND HSIEN WU.

From the Biochemistry Department, Medical College of Alabama, Birmingham.

Although the concentration of urea plus ammonia (U + A) in saliva is known on the average to approximate that in blood(1-5), the determination of U + A in saliva has not come into clinical use, probably on account of the wide variations encountered in individual samples.

Various methods for blood urea have been applied to saliva. Whatever the chemical technic used, the manner of collection of saliva samples is more important. In most previous studies, the volume of saliva required for analysis was relatively large, and, to obtain sufficient samples, artificial stimulation of the salivary glands by chewing paraffin was resorted to. The increased rate of flow due to

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stimulation tends to lower the salivary U + A, as Barnett and Bramkamp(6,7) have shown. On the other hand, slow-flowing saliva obtained without stimulation may give U + A values which are even higher than that of blood urea. This is probably due to contamination by products of bacterial action on food debris or on nitrogenous constituents, other than urea. in saliva itself(8). The contamination is greater when the time required for collection is prolonged.

Using Conway's micro-diffusion technic(9, 10) which has not been applied to saliva before, U + A can be determined with 1 ml or less of saliva. Sufficient saliva for duplicate analyses can be collected in a few minutes without artificial stimulation, and the chief cause of variation of the U + A value, as mentioned above, is thus removed.

Experimental. Collection of sample. Half an hour before the collection of a sample the teeth are brushed or the mouth thoroughly rinsed. The saliva is then swallowed continually for about 15 minutes to remove the remnant of the rinse water. One minute before the time of collection, the edge of a shortstem funnel (50 mm diameter) is pressed lightly against the lips. With mouth moderately open and head leaned forward, saliva is allowed to flow freely into a 10 ml graduate until about 2.5 ml have been collected. This requires only a few minutes. The moment when saliva actually begins to flow into the funnel is taken as the initial time of the collection. No sucking or spitting is necessary and should be avoided. Saliva collected in this way is usually water clear. During the collection of the sample, care is taken not to breathe through the mouth to avoid dilution of the sample by condensed moisture.

Determination of U + A. The determination of U + A is carried out in a 68 mm diffusion unit according to Conway. The procedure of Steinitz(11) was followed with slight modifications. Into the central compartment of the diffusion dish is measured 1 ml of a 2% boric acid solution containing an appropriate amount of methyl red + methylene blue as indicator. Into the outer compartment is delivered 1 ml of saliva and 0.5 ml of urease solution. The urease solution is prepared by dissolving eight urease tablets* in 10 ml water and adding 0.3 ml of a phosphate buffer solution (6.9 g of $NaH_2PO_4 \cdot H_2O$ and 17.9 g of $Na_2HPO_4 \cdot 12H_2O$ in 100 ml water). The urease is allowed to act on saliva for 10 minutes before adding 1 ml of saturated potassium carbonate. After 90 minutes the ammonia absorbed in the 2% boric acid solution is titrated with N/70 HCl from a 1 ml micro-burette.† For 1 ml of saliva, the amount of HCl required for titration ranged from 0.4 to 1.0 ml. Duplicate determinations usually agree within 0.01 ml of acid. The ammonia-N thus determined, after correction for blank, represents U + A nitrogen. The salivary ammonia-N which can be separately determined by the same procedure by replacing the urease with 0.5 ml of ammonia-free water usually amounts to 1-2 mg of N per 100 ml saliva. As little as 0.2 ml of saliva suffices for a determination. To minimize experimental errors, we chose to use 1 ml in our present study.

Results and discussion. The reproducibility of salivary U + A values is shown by experiments in which saliva samples were collected at frequent intervals. In one subject, samples collected at 10 minute intervals from 9:30 A.M. to 11:00 A.M. gave values ranging from 10 to 11.2 with an average of 10.6 ± 0.4 (S.D.) mg N per 100 ml of saliva. In another subject, samples taken every 15 minutes from 8:50 A.M. to 10:50 A.M. gave values ranging from 9.2 to 10.2 with an average of 9.7 ± 0.3 (S.D.) mg N per 100 ml saliva.

Table I shows some salivary U + A values

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* Made by Hynson, Westcott and Dunning, Inc., Baltimore. Each tablet contains 25 mg urease.

† This can be improvised by drawing the tip of a 1 ml graduated pipette to a capillary and attaching a 2 ml syringe to the upper end.

TABLE I. Salivary Urea in Normal Persons. Mg urea plus ammonia nitrogen per 100 ml saliva.

Subject, sex and age	Date	Time of collection										Approx. rate of flow, ml/hr
		6	8	9	10	11 A.M.	12 N.	1	2	3	4 P.M.	
H, M, 56	May 5				12.6		13.0		12.8		11.6	45
	" 6	12.8	12.6		10.8		12.8					
	" 11				11.1	15.1	11.9	15.0	14.6	13.9		
	" 12	11.5	10.9	12.8	11.7	11.7	12.2	10.1	10.9	12.6		
	Oct. 24		11.4	12.1	12.6	12.8	11.5	12.0	12.3	11.5		
Y, F, 48	June 7					12.4	14.3			15.3		25
	" 14			16.7	14.7	15.7	16.1			17.3	16.7	
	Oct. 25			10.3	10.4	12.1	11.4	11.3	10.1	10.4	10.4	
J, M, 32	June 16			17.6	18.1	15.0	15.9		16.4	18.6		30
	Oct. 23			13.1	14.5	11.9	12.8		11.8	13.6		
G, M, 30	June 22			11.4	9.1	8.7	9.0	8.8	11.3	9.5		30
S, M, 28	" 21				15.6	11.3	13.4	10.8	13.3	13.8		15
D, F, 22	" 8				9.9	11.1	11.2		10.3	11.7		12
	" 15			15.5	14.5	12.5	12.5		12.2	12.8		
R, M, 21	" 8				7.4	8.2	8.6		8.2	9.7		25
C, F, 20	" 9			11.3	11.1	11.3	11.3		9.8	11.0	10.2	20
V, M, 18	" 7					9.7	10.4		9.4	9.4		15
	" 9			9.2	11.1	11.7	9.9		8.4	9.7	10.4	

in normal adults. It will be noted that in some persons the U + A concentration is practically constant from hour to hour. In others, there is some variation which may reflect a variation of the urea concentration of the extracellular fluid after intake of food or drink. While the average level of U + A in the same person may change considerably from season to season (*e.g.* subjects J and Y), it remains the same from day to day in the same season (*e.g.* subjects, H, D, and V).

Whether the salivary U + A is exactly equal to blood urea or not, it has a significance of its own. Since the determination of U + A in saliva by the micro-diffusion method is so simple, it deserves consideration as a routine test in the clinical laboratory.

Becks and Wainwright(12) called attention to the fact that human saliva is a secretory and/or excretory product and that the rate of secretion of a substance as well as the concentration of the substance in saliva should be considered. Morris and Jersey(13) reported that the rate of urea secretion of the resting gland was more uniform than the concentration of urea. But this conclusion was not borne out by their data (Table III in their paper).

Since the calculation of the rate of salivary urea secretion requires a knowledge of the rate of flow which is much more variable than urea concentration and which cannot be accurately determined in short intervals, it seems better to express salivary U + A values simply in terms of concentration and not as rate of secretion.

Summary. Using Conway's micro-diffusion technic, urea plus ammonia determinations can be made with 1 ml or less of saliva. Sufficient saliva for duplicate analyses can be collected in a few minutes without artificial stimulation. Saliva collected in this way gives reproducible values in the same individual.

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Effect of Formaldehyde on Certain Oxidative Enzymes. (18413)

FREDERICK BERNHEIM.

From the Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, N. C.

Although formaldehyde is a highly toxic substance its effect on enzyme systems has apparently not been studied. In order to determine whether it was equally toxic to all enzymes, the oxidation of various compounds by a standard rat liver or kidney homogenate was studied in the presence of small amounts of formaldehyde. The results showed that the succinic acid, choline, sarcosine, and L-proline oxidases were inhibited by formaldehyde in concentrations which were without effect on the cytochrome, amine, and D-amino acid oxidases.

Experimental. Liver or kidney homogenates were made by grinding the tissue in a mortar with 0.05 M Na-K-phosphate buffer pH 7.8, 1.0 ml per 0.5 g tissue, and straining through muslin. 0.5 ml of the resultant suspension was placed in each Warburg flask which had a final volume of 2.0 ml made up of buffer and substrate. The latter was added either before or after the formaldehyde in order to see whether it protected the enzyme. Saturation concentrations of each substrate were used. All the enzymes tested have pH optima at or near pH 7.8.

Formaldehyde acts rapidly. Immediately after the addition of 6.2 x 10^{-3} M to the liver suspension the rate of oxygen uptake is reduced 40-60%. Even though the homogenate was made in a standard way variations in the percentage inhibition occurred. As a basis for the following comparison the inhibition of the succinoxidase was used. In the presence of the above concentration of formaldehyde added before the substrate the succinoxidase of liver was, in this instance, inhibited 42%, the choline oxidase 75%, L-proline oxidase 74%, sarcosine oxidase 30% and tyramine and cytochrome oxidases 0%. In the kidney when the succinoxidase was inhibited 80% there was no inhibition of the oxidation of D-methionine or D-alanine. When tyramine, D-alanine or p-phenylene diamine were added to the succinic acid, the inhibition of the succinoxidase still occurred which indicated that these compounds were not preventing the formaldehyde from reacting with the respective enzymes.

TABLE I. Effect of 6.2, 9.3, and 12.4 × 10^{-3} M Formaldehyde Added 5 Minutes Before and After 4.2 × 10^{-2} M Sodium Succinate on the Oxidation of the Latter by Rat Liver Homogenate at pH 7.8, 37°. The succinoxidase activity under these conditions was approximately 100 μl O_2 per 10 min.

	% inhibition					
	6.2 × 10^{-3} M formaldehyde		9.3 × 10^{-3} M formaldehyde		12.4 × 10^{-3} M formaldehyde	
Time, min.	Before succinate	After succinate	Before succinate	After succinate	Before succinate	After succinate
0-5	16	0	34	0	57	0
10-15	14	0	30	10	48	18
20-25	18	6	31	20	47	29
50-60	21	21	35	34	49	42

Table I shows the effect of different concentrations of formaldehyde on the succinoxidase of liver and the effect of adding the succinic acid before and after the formaldehyde. If added before, the substrate protects the enzyme for a short time from the formaldehyde. The affinity of the latter for the enzyme is, however, so great that it displaces the succinic acid regardless of the concentration of the latter. Fumaric acid offers no protection. Similar results were obtained with the other enzymes.

In the rat liver, the order of the oxidation rates of the various added compounds was p-phenylene diamine 1, succinic acid 2, choline 3, L-proline 4, tyramine 5, sarcosine 6. In the kidney the succinoxidase was more than twice as active as the D-amino acid oxidase. It is obvious therefore the observed effects of formaldehyde are not correlated with the relative amounts of the enzymes present in the homogenates. Either the amino group of various enzymes have different affinities for formaldehyde or these groups are not important for the action of the three enzymes which are not affected by the reagent. Since doubling the formaldehyde concentration still does not inhibit these three, it is probable that the latter explanation is correct. Finally it may be mentioned that the formaldehyde inhibition of the succinoxidase is the same at pH 6.0 as it is at pH 8.0.

Summary. 1) Formaldehyde inhibits the succinic acid, choline, L-proline and sarcosine oxidases in liver homogenates. In equal concentrations, it has no effect on the amine and cytochrome oxidase of liver or the D-amino acid oxidase of kidney. 2) The prior addition of substrate protects the enzymes from the formaldehyde for a short period of time.

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Can Experimental Shock be Induced by Coronary Occlusion?* (18414)

Sherman Kupfer.† (Introduced by Carl J. Wiggers.)

From Western Reserve University School of Medicine, Department of Physiology, Cleveland, O.

Objectives of the Experimental Approach. Of the several hazards of coronary occlusion, the clinical development of a shocklike state has been discussed a good deal in recent years. The clinical inference that this is due to supervention of peripheral circulatory failure has not, however, received valid support. Experimental work appears to favor the conclusion that circulatory failure not attributable to severe irregularity of the heart beat is due successively to defection of useful contractions in the ischemic area, loss of total contractile energy through expansion of the affected area, and failure of the still viable fractions to compensate adequately(1-4). However, such conclusions are based on experiments extending only an hour or so beyond the time of coronary occlusion, after which the animals usually die due to ventricular fibrillation. Thus the most complete dynamic study of changes in intraventricular and aortic pressure pulses, that of Orias(2), stressed chiefly (a) that occlusion of the ramus descendens anterior is followed immediately by hypodynamic beats with shortened systoles, but (b) that these effects are usually compensated within 4 to 7 minutes through increase in initial tension in the left ventricle, which restores aortic and left ventricular pressures to normal levels. This compensation failed to occur in only three of 23 experiments and in these rapid dilation and failure of the heart supervened. The subject has also been studied on survival animals of which the report by Gross and his associates(5) may serve as an illustration. These investigators found, in dogs that survived for a day or more, a decrease in cardiac output, some irregular decline of arterial pressure, and evidence of pulmonary congestion based on a prolonged cyanide time. Since they were unable to establish any reduction in blood volume or fall in peripheral venous pressures they concluded that evidence for peripheral circulatory failure was lacking.

Despite the negative character of previous experimental work it seemed important that another attempt be made to determine whether hemodynamic signs of peripheral circulatory failure develop in animals which have survived occlusion of the ramus descendens anterior for 5 hours or more and in which serious arrhythmia has been prevented or mini-

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† Postdoctorate Research Fellow, National Heart Institute, U. S. Public Health Service.

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mized as a contributory factor to the circulatory failure.

Methods and Technics. Dogs anesthetized with 3 mg/kg of morphine and 200 mg/kg sodium barbital were used. The chest was opened and the heart exposed. Right and left atrial and aortic pressures were recorded by calibrated Gregg type manometers of suitable sensitivity and adequate frequency. Following the surgical procedures and cannulations, the animals were allowed to stabilize for an hour before occlusion of the ramus descendens anterior was attempted. In some animals control blood volume determinations or cardiac output determinations by the Fick method were made during this period, followed by subsequent determinations during later phases of the experiment, as described below. Standard technics were used for all of the foregoing procedures. Since the supervention of long periods of marked arrhythmia, such as frequently follow coronary ligation in dogs, can itself lead to an imbalance of the circulation which reduces cardiac output and arterial pressures, it was obviously important to prevent such irregularities if the development of myocardial or peripheral circulatory failure was to be assessed. Several expedients proved helpful in warding off such attacks in approximately half of the dogs used. Care was exercised to avoid accidental mechanical stimulation of the ventricles which are supersensitive and often respond by runs of premature systoles. The method of partial occlusion followed by complete occlusion in 30 minutes described by Harris(6) was found only moderately successful. Better results were usually obtained by intravenous administration of quinidine sulfate, 0.5 mg/kg. In earlier experiments quinidine was administered as soon as any ectopic beats appeared; subsequently, the drug was given about 15 minutes prior to coronary occlusion. When slowly given, such doses had no demonstrable effects on atrial or aortic pressure.

Results. Through adoption of the foregoing expedients, 11 experiments were realized in which irregularities were successfully avoided or were very transient in nature. In these, the hemodynamics due to myocardial or peripheral circulatory default could be studied for 5 to 7 hours after ligation of the ramus descendens anterior. Since other shock-provoking agents used in equivalent experiments on similarly anesthetized dogs with exposed hearts always cause irreversible failure involving the peripheral circulation in less than five hours, such a time span seemed sufficient for hemodynamic evidence of such failure to become manifest. Contrary to expectations, only one of the animals studied showed evidences of serious circulatory failure.

Blood volume and cardiac output changes. Blood volume determinations were made by the Evans' blue technic in all of the 11 animals during the preliminary stabilization period and again at the close of the experiment, 6 to 7 hours after coronary occlusion. In none of these were significant changes found. In 5 dogs cardiac output determinations were made by the direct Fick method during the control period, 45 to 60 minutes after ligation and again 5 hours after ligation. Comparisons were also made with output evaluations by the pulse contour method(7, 8). These results are summarized in Table I. In the first 2 experiments in which aortic pressures and pulse forms remained normal, no significant reduction in cardiac output (Fick) was evident. In the third experiment listed, significant reduction in cardiac output (Fick) supervened toward the close of the experiment owing to the supervention of ventricular alternation. In the fourth experiment listed, alternans of varying degrees occurred throughout the observation periods; the early reduction of cardiac output, however, did not persist; the output volume recovered at the end of 4 hours. In the fifth experiment listed, significant hypotension had developed at the end of a 5½ hour period, and cardiac output was decreased.

In order to compare changes in cardiac output determined by the Fick method with those of pulse contour method under normal

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TABLE I. Cardiac Output in Liters/min./sq.m. Before and After Coronary Occlusion.

Exp. No.	Fick	Terminal changes	Pulse contour method	Terminal changes	Time in relation to coronary ligation
13*	2.88		2.24		Control
	2.39		1.82		1 hr
	2.68	—0.20	2.01	—0.23	5 hr
31	2.39		3.25		Control
	2.34		2.78		30 min.
	2.26	—0.13	4.12	+0.87	4½ hr
27	1.94		2.28		Control
	1.32		1.81		1 hr
	0.88†	—1.06	1.75	—0.53	5 hr
19	2.38		2.20		Control
	1.98†		2.13†		45 min.†
	2.05†	—0.33	2.10†	—0.10	4 hr†
24	1.98		3.84		Control
	1.07		2.52		45 min.
	1.18	—0.80	3.43	—0.41	5½ hr (hypotension)
21	1.82		1.78		Control
22	2.67		1.74		”
26	2.12		2.20		”
12*	1.55		3.25		”
	2.40	+0.85	3.40	+ .15	Post-ligation
H-F 1	1.30		1.34		Control
	1.64		1.52		”

* A-V oxygen differences determined in duplicate by Scholander method, checking by 0.2 vol. %—others in duplicate by method of Peters and Van Slyke, checking within 0.1 vol. %.

† *Pulsus alternans*, spontaneous.

conditions, 5 other experiments are added in Table I in which observations could not be completed owing to supervention of fibrillation. Excellent agreement in estimation of control cardiac outputs is noted in Exp. 19, 21, 26, and HF-1 in which the pulse contour method yielded outputs deviating from Fick determinations by only +.08 to –.18 L./min. In the other six control determinations the deviations of the pulse contour values ranged from –.093 (Exp. 22) to + 1.86 (Exp. 24) L./min. Such deviations support the conclusion of previous work from this laboratory (9) that the method of Hamilton and Remington cannot be relied upon for quantitative estimations of cardiac output and calculation of TPR even under normal conditions. In Exp. 12, 13, 19, 24, and 27, in which normal pressure ranges and contours were maintained, the directional changes in cardiac output determined by the pulse contour method corresponded qualitatively. However, in Exp. 31 the quantitative and directional changes deviated considerably.

Aortic and Atrial Pressure Changes. The segments of original records in Fig. 1 serve to show the nature of the left and right atrial pressure changes up to 7 hours after ligation of the left anterior descending ramus and also indicate in segment B the method used in measurement of atrial pressures. Measurements were made (a) of the instantaneous pressures at the end of diastole (Z point) which should be equivalent to the initial pressures in the ventricles, and (b) of the pressure near the end of systole (V point) which is a criterion of the extent to which the atria fill during ventricular systole. (For details see Little *et al.*) (10). All such measurements were made at comparable phases of respiration and the illustrative records represent such selected pressure pulses. In Fig. 1, segment A represents a record taken at the end of the one hour control period. Segments B to D illustrate the pressure changes 1, 30 and 60 minutes after coronary occlusion. Obviously, records taken immediately after coronary occlusion show that systolic, diastolic, and pulse pressures decrease somewhat, the aortic pressure rises more gradually, and the

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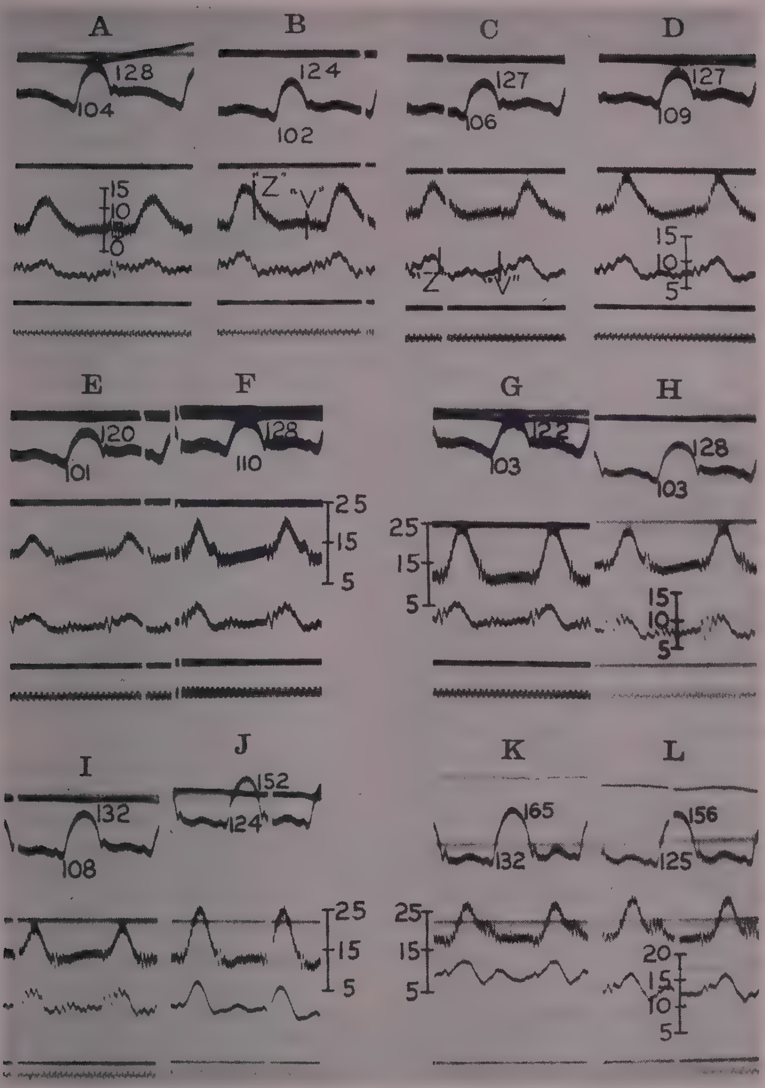

Fig. 1.

Segments of records showing changes in aortic (upper), left atrial (middle), and right atrial (lower) pressures before (A) and at various times after coronary ligation. Post-ligation times: B, 1 min.; C, 30 min.; D, 60 min.; E, 2 hours; F, 3 hours; G, 4 hours; H, 5 hours; I, 6 hours; J, 7 hours; R, 2 min. after infusion; L, 5 min. after infusion. Calibration scales for aortic pressure in mm Hg; for atrial pressures in cm saline. Discussion in text.

systolic ejection is abbreviated. It requires no calculations to state with assurance that systolic discharge is immediately reduced and with it cardiac output/min., despite the slight cardiac acceleration. There is a rather prompt recovery from this hypodynamic state of the left ventricle and at the end of an hour (segment C) aortic pressure pulses and the duration of systolic ejection have resumed preligation shapes and values. All of these observations during the first hour accord with the findings of Orias reported from this laboratory(2). Our chief interest focuses in the changes in atrial pressures during this period. It may be noted that left atrial pressure rises almost immediately and continues to increase both at the Z and V points, whereas pressure in the right atrium shows no significant changes.

Segments E, F, and G illustrate the dynamic conditions at the end of the second, third, and fourth hours respectively. Arterial pressures are relatively well maintained but the pulse pressure is reduced slightly, and the pressure pulse displays a slower rise and more rounded contour. However, in segments H, I and J the pressure pulses indicate continued improvement in systolic discharge. In fact, 7 hours after ligation (J) arterial pressures and pressure pulses exceed those during the control period (A). The only similarity to pressure pulses observed with low systolic discharge in shock is the flattening of the diastolic limb; but its significance has never been satisfactorily clarified. Considerable unexplained variation takes place in the amplitude of the atrial wave (*cf.* segment E and I with G, H, J). However, Z and V pressures proved to be affected very little by these variations. All records revealed persistence of an elevated atrial pressure. These changes conform to the concept that compensation by viable myocardium of the left ventricle is achieved through an increase in atrial filling pressure and a resultant stretch of the left ventricle.

In order to determine whether the limit of cardiac competency to increased filling and stretch was approaching a limit at the end of 7 hours, a femoral infusion of 200 cc of blood was given within 2 minutes. Segments K and L depict the reactions 2 to 5 minutes after completion of the infusion, and the graph of Fig. 2 shows the pressure changes. The aortic pressure pulses definitely show that systolic discharge was augmented; however, the flattened diastolic limb and larger dicrotic wave persist. Pressures are immediately elevated at the Z and V points in both atria (K), but while right atrial pressure has declined within 5 minutes, left atrial pressure remains elevated (J), indicating that the ability of the left heart to respond to increased stretch has not been exceeded as a result of the infarction. However, as indicated in the graphs of Fig. 2, ventricular alteration was induced. It may be added parenthetically that had right atrial pressure alone been recorded, the inference might have been drawn that the maintenance of the systolic discharge of the left ventricle bears no relation to atrial pressure, and that therefore Starling's law is inapplicable. Cardiologists who have drawn such conclusions on the basis of catheterization studies may have fallen into similar traps.

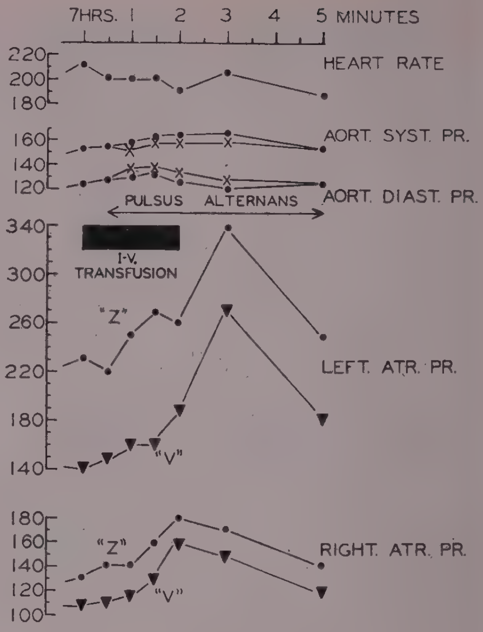

FIG. 2.
Graphs showing minimal changes in heart rate/min. and pressures during and after transfusion of same animal, as shown in Fig. 1. Scales as in Fig. 1.

The foregoing types of reactions in which left atrial pressures were elevated promptly after ligation and remained high, while right atrial pressures continued near normal levels, was characteristic of six of these experiments. In only one of these experiments, illustrated in Fig. 3, did signs of circulatory failure, characterized by reduction in systolic, diastolic, and pulse pressures, supervene after the

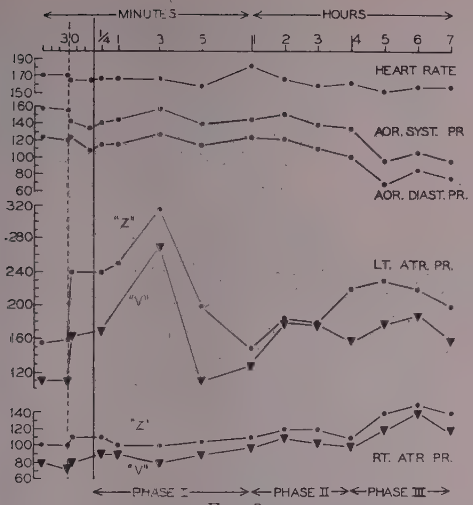

Fig. 3.
Plot of data showing changes in heart rate/min., aortic and atrial pressures after coronary occlusion. Scales as in Fig. 1. Discussion in text.

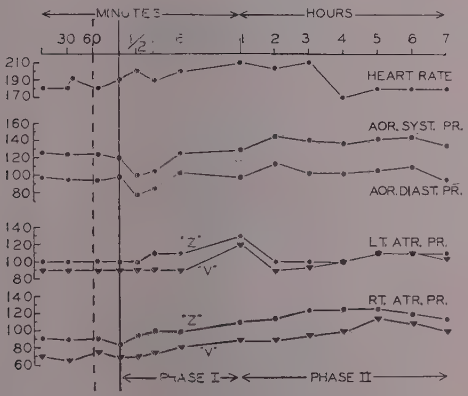

Fig. 4.
Plot of data showing changes in heart rate/min., aortic and atrial pressures after coronary occlusion. Scales as in Fig. 1. Discussion in text.

third hour (Phase III). Since this was accompanied by a marked increase both in right and left atrial pressures, the inference is that failure was of cardiac and not of peripheral origin.

In 5 other experiments pressures in the 2 atria followed a different trend. Left atrial pressure either returned to control levels after an initial increase, or displayed no initial elevation and only a delayed slow increase. Figure 4 illustrates such a lag in the rise of left atrial pressure immediately following ligation, but at the end of an hour it was definitely elevated. In these cases right atrial pressure increased progressively from the start, indicating that reduction of venous return characteristic of shock could not be involved. On the contrary, under the stimulating influence of the augmenting atrial pressures and increased diastolic stretch, the right ventricle maintained an adequate return to the left ventricle and an adequate systolic discharge, as evidenced by the maintenance of good aortic pressure and an increase in pulse pressure.

Discussion. No lengthy discussion of the results presented is required. Blood volume determinations in 11 animals showed no significant changes from control values. Cardiac output was reduced only in animals when they developed ventricular alternation. The fact that right atrial pressures did not decrease at any time up to seven hours indicates that venous return is not decreased. Hence there was no evidence that circulatory failure of peripheral origin supervened. With a single exception, aortic pressure pulses also gave no evidence that the output of the left ventricle was impaired even when submitted to added strain of transfusion.

The results presented indicate clearly that changes in the dynamics of the left ventricle cannot always be gauged by measurement of pressure in the right atrium. Furthermore, it is quite generally recognized that alterations in peripheral venous pressure may be wholly misleading, for they do not necessarily correspond to absolute changes in right atrial pressures.

Summary and conclusions. The left ramus descendens anterior of the exposed hearts of dogs was ligated, arrhythmia was prevented by use of small doses of quinidine, and records of aortic, left atrial and right atrial pressures were repeatedly recorded by calibrated Gregg-type manometers for 5 to 7 hours. Blood volume and cardiac output determinations were made in some animals. The results indicate that up to 7 hours there is no evidence, on the basis of an analysis of cardiac output, blood volume and pressure pulses, that circulatory failure of peripheral origin supervenes in dogs after ligation of a major coronary branch.

Comparison of cardiac output data obtained by the Fick method and the aortic pulse contour method showed sufficient disagreement to indicate that the latter method is not sufficiently accurate for quantitative evaluation of cardiac output, even when pressure pulses of normal contour and normal aortic pressures prevail.

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Effect of Adding Carbon Dioxide to Inspired Air on Consciousness Time of Man at Altitude.* (18415)

F. G. HALL AND K. D. HALL.

From the Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, N. C.

Accurate appraisal of the factors which influence useful consciousness states in flyers under hypoxic stress is of fundamental importance in aviation. The duration of consciousness of subjects breathing ambient air of normal composition at various altitudes has been reported by several investigators (1-3). A method which gives a critical end point for loss of useful consciousness has also been described(4). The following reports extend the range of studies to include the addition of carbon dioxide to the normal composition of inspired air. Such a situation may at times exist in aircraft flying at high altitudes.

It is apparent from the paper by Fenn *et al.* (5) that the additions of carbon dioxide to inspired air containing nitrogen will have a beneficial effect on subjects at altitude. Otis *et al.*(6) have shown, however, that carbon dioxide added to inspired oxygen does not increase the tolerance of mice and men at altitude. Because of the complexity of the physiological processes involved one cannot safely predict as to the quantitative effect of carbon dioxide on consciousness time. Consequently, an experiment was designed to determine the effect of certain concentration of carbon dioxide on the duration of consciousness at 30,000 (226 mm Hg) and 35,000 feet (179 mm Hg) simulated altitudes.

Procedure. The time of useful consciousness was determined under conditions where the ambient pressure in a decompression chamber was maintained constant and the subjects changed from breathing pure oxygen to breathing gas mixtures from Douglas bags. Gas mixtures were made in a large spirometer and transferred to Douglas bags just previous to ascent. It should be noted that the partial pressure of oxygen in the inspired air was kept constant and that the addition of carbon dioxide was not made at the expense of oxygen. In other words a calculated quantity of oxygen was added with the carbon dioxide to correct for the dilution effect. Analyses during experiments confirmed the accuracy of the gas mixing procedure. Thus uniform partial pressure of oxygen existed in the inspired air of both control and experimental tests. Gas analyses were made in 2 ways. Samples of air delivered to the mask were analyzed by means of a Haldane gas analyser. A Pauling oxygen meter was also employed to determine the partial pressure of oxygen which was kept constant within ± 1 mm Hg. Carbon dioxide analyses showed that the CO_2 concentration in the experimental tests ranged from 13.8% to 14.3% at 30,000 feet and 18.8% to 19.2% at 35,000 feet. Standard Air Force oxygen masks were used and a three way valve was arranged to connect the oxygen supply, or the Douglas bags, to the mask. Mask leakage was inappreciable. Breathing resistance was kept at a low value. Gas samples were drawn from the oxygen mask inlet and analyzed for composition of the inspired air. The effective external dead air space of the oxygen mask was approximately 90 cc. Nine healthy young men, aged 20-24, were selected as subjects. Each was given preliminary training and experience in high altitude ascends in the decompression chamber. No subject showed any untoward symptoms resulting from these experiments. Resuscitation following loss of consciousness was prompt and without any detectable after effects, either from hypoxia or carbon dioxide breathing.

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* Work done under contract with the Physiology Branch, Aero-Medical Laboratory Materiel Command, U.S.A.F.

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Each subject served as his own control. This is necessary since a marked individual variation occurs in consciousness time at altitude. Two tests were made on each subject with a 40-50 minutes interval of rest between runs. On one day the control run was made first and on the next day the experimental run was made first. Subjects were not informed as to which were control runs and which were experimental runs and in only a few cases were they able to detect the one to which they were being subjected. Three control and 3 experimental tests were performed for each subject. The method for measuring the duration of useful consciousness at altitude has been described by Hall(4). Photokymographic records were used to determine pulmonary ventilation from which ventilation ratios were calculated.

Results. Summaries of results are presented in Tables I and II. It will be noted that a marked difference in useful consciousness time at simulated altitudes of 30,000 (225 mm Hg) and 35,000 feet (179 mm Hg) occurs when certain amounts of carbon dioxide are added to the inspired air. This effect is more marked at 30,000 feet. It is probably that the effect of addition of carbon dioxide to inspired air will have a less pronounced effect at higher altitudes.

TABLE I.

	Consciousness time at 30,000 ft. breathing	
Subjects	Ambient air (normal composition) sec.	Gas mixture containing 14% CO_2, 21% O_2, 65% N_2, sec.
H	96	181
T	87	198
TK	91	126
P	90	110
D	78	177
J	79	137
B	73	181
JW	95	172
K	108	184
Avg	88	163

TABLE II.

	Consciousness time at 35,000 ft. breathing	
Subjects	Ambient air (normal composition) sec.	Gas mixture containing 19% CO_2, 21% O_2, 60% N_2, sec.
H	60	82
R	60	67
T	58	83
TK	50	66
D	54	67
J	45	66
B	54	66
JW	56	64
K	59	69
Avg	55	70

In Fig. 1 and 2, pulmonary ventilation ratios are shown. These were calculated from photokymographic records of the breathing patterns (Rheopneumograms) made during the simulated flights. A ventilation ratio of 1 indicates the level of the pulmonary ventilation of subjects breathing pure oxygen for a period of two minutes just previous to breathing ambient air. Records were made on all subjects for all tests and the values indicate the mean values for all tests. Thus on each graph is shown the effect of the "anoxia drive" on pulmonary ventilation when subjects are breathing ambient air at low barometric pressures and the effect of supplementing this air with carbon dioxide. It should be pointed out that the quantity of carbon dioxide added does not raise its partial pressure in the inspired air above the normal value of 40 mm Hg for alveolar air. Consequently, the respiratory center maintains a quantitative regulation of pulmonary ventilation

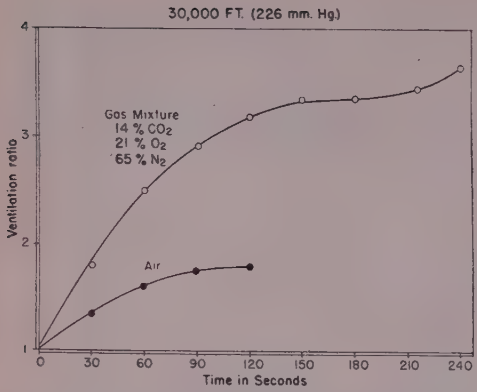

FIG. 1.

Ventilation ratios of subjects breathing air of normal composition and a gas mixture containing 14% carbon dioxide, 21% oxygen, 65% nitrogen, at a simulated altitude of 30,000 feet.

throughout the duration of consciousness in these experiments.

Doubtless there are many interacting factors involved in the explanation of these results. Carbon dioxide through its influence on cerebral blood vessels may influence the supply of oxygen to the brain. It seems plausible from theoretical consideration presented by Fenn *et al.*(5), that the more important factor is the high ventilation rate caused by the increased carbon dioxide and resulting in an increased partial pressure of oxygen in alveolar air, when nitrogen is a component of the inspired air. Consequently, carbon dioxide increases tolerance to altitude when breathed with normal air and does not do so when breathed with pure oxygen. Further experiments are necessary to place these factors in their proper relationship.

Summary. The addition of certain amounts of carbon dioxide to ambient air at low barometric pressures of 225 mm Hg, and 179 mm Hg increases the duration of useful consciousness in human subjects. This effect is more pronounced at lower simulated altitudes than at higher altitudes.

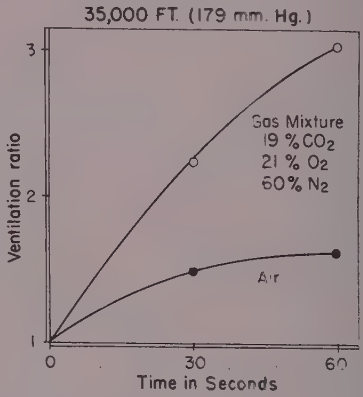

FIG. 2.

Ventilation ratios of subjects breathing air of normal composition and a gas mixture containing 19% carbon dioxide, 21% oxygen, and 60% nitrogen, at a simulated altitude of 35,000 feet.

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Nutritional Hydrocephalus in Infant Rats.* (18416)

L. R. RICHARDSON.

From the Department of Biochemistry and Nutrition, Texas Agric. Exp. Station, Texas Agric. and Mechanical College System, College Station, Texas.

Hydrocephalus in infant rats as a result of inadequate nutrition of the mother has been reported by Richardson and Hogan(1) and by Richardson and DeMottier(2). Recently Hogan *et al.*(3) reported that folic acid decreased the incidence of the abnormality and

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* The author is indebted to Merck and Co. for vitamins and APF No. 3, to Lederle Laboratories for folic acid and APF concentrate, to Wilson Laboratories for supplying liver "L," and to Nancy Muller and Christine Lovelace for technical assistance.

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TABLE I. Incidence of Hydrocephalus in the Young Born to Hydrocephalic Parents.

Mating	Diet	No. females	No. litters	No. of young Born, No.	Hydrocephalic No.	%
Hyd. M x Normal F	Basal	4	6	32	3	9.48
Hyd. M x Normal F	Stock	2	6	36	0	0
Hyd. M x Hyd. F	,,	2	3	25	0	0
Normal M x Hyd. F	,,	1	2	20	0	0

that the addition of crude folic acid antagonist to the basal diet of the mother increased its incidence in young.

Two hydrocephalic males and females each were reared to maturity in this laboratory, and litters were obtained by mating these animals to each other and to normal animals. Studies have been carried out also on the effect of folic acid and APF concentrates in the diet of the mother on the incidence of hydrocephalus in the young. The results of these studies are described in this report.

Experimental. A. *Basal diet and care of animals.* The composition of the basal diet was the same as that described by Richardson and Hogan(1) with the exception that 4% of Wesson Oil was used instead of lard and Labco Vitamin-free casein was used as a source of protein. The females received the experimental diet from weaning. Five females and a male were kept in a 3 unit Wahmann cage with 2 mesh galvanized wire cloth on front and bottom. When a female was pregnant as shown by a marked increase in weight, she was transferred to a 2 unit cage with 3 mesh wire cloth. Shredded paper was used as a source of bedding for 4 to 5 days after the birth of the litter. The laboratory was controlled so that the temperature did not go above 82° or below 75°F.

B. *Observations on the offspring of hydrocephalic parents.* During the course of the investigation 2 hydrocephalic males and females each were reared to maturity. These 4 hydrocephalic rats were born at about the same time, and they were used to study the effect of heredity as well as diet on the incidence of hydrocephalus. These data are summarized in Table I. After a total of 15 litters were produced the 2 males and one female were killed, and autopsy showed that they had severe hydrocephalus. The other female was mated to a normal male and produced 2 additional litters of normal young. This female weaned 3 litters containing 24 normal young. She was killed and autopsy showed that she had severe hydrocephalus.

TABLE II. Effect of Diet of Mother on Incidence of Hydrocephalus in Infant Rats.

Group No.	Supplement	Young observed Born, No.	Hydrocephalic No.	%
1	None	1604	120	7.48
2	Folic acid*	1254	2	0.15
3	Merck APF No. 3†	890	18	2.02
4	Folic acid* Merck APF No. 3†	761	1	0.13
5	Folic acid* Lederle APF 1.0%	288	2	0.69
6	Folic acid* Liver "L" 3.0%	719	0	0.00

* 0.2 mg per 100 g diet.
† 2 g per k of diet.

C. *Folic acid, APF concentrates, liver extracts, and the incidence of hydrocephalus.* Data showing the effect of folic acid, liver "L," an APF concentrate and combinations of folic acid and APF concentrates in the diet of the mother on the incidence of hydrocephalus in the offspring are given in Table II. There it is seen that both folic acid and the APF concentrate decreased the incidence of hydrocephalus. When the mothers received both folic acid and an APF concentrate, the incidence was essentially the same as when they received folic acid alone. None of the young from mothers which received folic acid and liver "L" developed the abnormality.

D. *Folic acid antagonist and hydrocephalus.* Fifteen females were reared from weaning on the basal diet supplemented with 2 g of APF concentrate No. 3 per kilo. At the time the females were mated, 10 mg per kilo of a crude methyl folic acid antagonist were added to the diet. A total of 25 litters containing 125 young were born within the first 5 month period. Six litters containing 29 young were weaned. One young had hydrocephalus. No additional litters were produced within a second 3-month period, and the observations were discontinued. The folic acid antagonist under these conditions decreased the number of litters produced per female but it did not increase the incidence of hydrocephalus.

Discussion. Only a small number of animals were available for studies on reproduction with hydrocephalic rats, but the observations have special significance for two reasons. First, as far as the author is aware no studies on reproduction in animals or man with any type of hydrocephalus have been reported previously. The data show that rats were able to produce and rear normal young when they received a stock diet, even though both parents had severe hydrocephalus. When they received a basal diet low in folic acid, the incidence was about the same as when normal parents received this diet. Second, the possibility that heredity may be a factor in producing this type of hydrocephalus has never been eliminated. The data available, although not conclusive, suggest strongly that heredity has very little or no influence. The observations of Hogan *et al.*(3) that the addition of folic acid to the diet of the mothers decreased the incidence of hydrocephalus was confirmed. The incidence was less also when Merck's APF concentrate was added, and the activity of the concentrate did not appear to be due to the folic acid which it supplied. The basal diet and the concentrate were assayed for folic acid by the procedure described by Flynn(4) and the folic acid content was increased very little by the addition of 2 g of the concentrate per kilo of diet. The increase was from 0.090 to 0.096 μg per gram. Further evidence that the activity of the concentrate was due to some other factor, is shown by the fact that the addition of crude methyl folic acid to the diet containing the APF concentrate did not increase the incidence of hydrocephalus. Schaefer *et al.*(5) have reported that either folic acid or vitamin B_{12} afforded some protection of rats against renal damage, and both vitamins appeared capable of replacing each other for normal hemoglobin production. A similar effect of vit. B_{12} on hydrocephalus could account for the folic acid antagonist not increasing the incidence of hydrocephalus when the diet contained the APF concentrate.

Conclusions. Mature hydrocephalic parents were capable of producing young and when they received a stock diet none of the offspring were hydrocephalic even though both parents had severe hydrocephalus. In studies on the effect of folic acid and APF concentrates on the incidence of hydrocephalus, 7.48% of the young born to female rats, receiving a purified diet low in folic acid developed the abnormality. The incidence was decreased to 0.15% by the addition of 100 μg of folic acid per 100 g of diet. It was 2.02% when the diet contained 2 g of Merck's APF concentrate No. 3 per kilo. When both folic acid and Merck's or Lederle APF concentrates were added, the incidence was essentially the same as when folic acid alone was added. None of the young from parents that received liver "L" was hydrocephalic. When 10 mg of crude folic acid antagonist were added to the basal diet which contained Merck's APF concentrate, the number of litters per female were decreased, but there was no increase in the incidence of hydrocephalus.

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Extraction of T-1824 in the Presence of Gross Hemolysis and Lipemia.
(18417)

THOMAS H. ALLEN.

From the Department of Physiology, College of Physicians and Surgeons, Columbia University.

Those who use the dye-hematocrit method (1) find that its many advantages are nullified by hemolysis and lipemia(2). For this reason a number of procedures have been devised for the more or less separate measurement of dye(3-8). The sorption of the blue dye, T-1824, has been described(9) as this occurs on aluminum and magnesium oxides, but the recovery is inconsistent largely because of instability at very low pH.*

T-1824 has been separated from plasma by adding an excess of anionic detergent and extracting with a Cellophane foil in which the optical density was measured(10). The dye was stable, but the routine was laborious. Instead of small foils a column of shredded paper can be used. The dye is sorbed on the column, thoroughly rinsed, then eluted and measured with ease.

Materials and procedures. The sorption column can be prepared from almost any type of cellulose. A dozen sheets of tissue paper† are shredded by brief agitation in a Waring Blendor containing 0.9 g% sodium chloride. The pulp is stored in a flask from which portions are transferred to Büchner funnels having a floor made of a coarse fritted glass disc. The pulp fiber is firmly but not forcibly packed with a plunger, only the excess fluid is drained through and then the head is levelled off by gentle tamping. The column—1.0 cm in length, 1.2 cm in diameter and 0.13 g in dry weight—is ready to receive the dye-tinged plasma which more than 5 minutes previously has been mixed with one-half of its volume of 15 g% sodium "Lorol" sulfate PT.‡ The mixture is either left to drain slowly or else sucked through in about 7 minutes. The test tube and column are rapidly rinsed with 3 successive 2 ml portions of 0.9 g% sodium chloride. Just as the last of the rinse is receding into the column, the funnel is removed from the aspirator flask and inserted into a 10.0 ml volumetric flask. The dye is eluted with 9.5 ml of a 1:1 volume mixture of acetone and distilled water. The first 3 to 4 ml are rapidly forced through by air pressure supplied from a reservoir flask connected through check valves to a hand-bulb pump. During the next one-half or more hours eluent is allowed to seep through the column. The remainder is then forced through, brought to volume, and is ready for measurement of optical density using a Beckman spectrophotometer.

In order to test for consistency of extraction it is necessary to know the purity of the T-1824 preparation. The aqueous solution of dye in lot no. 7 ampules§ contains 4.51 mg dry weight per ml. In this solution there is

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[*] Observed by J. C. Williams, first year medical student working in this laboratory on a summer research fellowship.

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[†] Cellulose Wipes, Johnson and Johnson.

[‡] "Recrystallized from technical grade of sodium dodecyl sulfate made from a mixture of alcohols—contains some sodium tetradecyl sulfate and less than 0.2% of either sodium chloride, sodium sulfate, or unreacted alcohols," Fine Chemicals Division, E. I. du Pont de Nemours and Co.

[§] Furnished by the Warner Institute for Therapeutic Research, New York City.

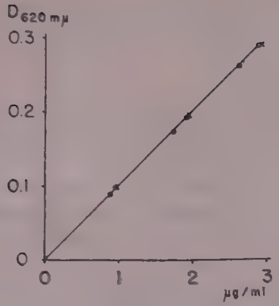

FIG. 1.

Optical density at 620 mμ of various concentrations in μg T-1824 per ml, not corrected for 0.5% impurity. *Crosses*, in 1:1:acetone:water; *circles*, in acetone:water containing 5 volumes % of 0.9 g % sodium chloride. Cuvette depth, 1.000 cm. Spectral interval, 4.8 mμ.

a red impurity(11) which washes through in the rinses and accounts for only 0.49% of the maximum absorption of light. The remainder is accounted for by the T-1824 which is eluted by 30 ml of acetone: water. The ampuled T-1824 when diluted in acetone:water has a mean optical density of 0.1005 per μg per ml. This result was obtained by using an accurate means of making known dilutions. A drop of the T-1824 solution was weighed to the nearest 0.1 mg in a volumetric flask and then brought to the 10.0 ml mark by addition of acetone:water. Three volumetric dilutions were prepared from each of three flasks. The optical density of the various concentrations is illustrated in Fig. 1. It is also shown that the optical density is not affected by the presence of 5 volumes % of saline, which corresponds to the relative volume in the effluent. Since 0.5% of the optical density is caused by impurity, it is concluded that in a 1.000 cm cuvette 1.00 μg of T-1824 per ml of effluent has an optical density of 0.100 at 620 mμ wave length of light with a spectral interval of 4.8 mμ. The variance of measurement has a standard deviation of 0.0011.

Recovery from "clear" plasma. Small weighed amounts of the stock solution of dye were brought to a volume of 10.0 ml by addition of plasma in which before mixture the optical density was 0.025 in the dog plasma and 0.048 in the human plasma. The concentration of dye after correction for impurity was 21.20 μg per ml in dog plasma and 18.08 μg per ml in human plasma. An accurately graduated pipette was used in transferring small volumes, 0.10 ml to 2.00 ml of the dye-tinged plasma to test tubes containing one-half these volumes of 15 g% "Lorol" sulfate. Nine or 10 determinations were made on each solution; and the recovery of various amounts is shown by the circles in Fig. 2.

Recovery from lipemic plasma. Several hours after the subject had consumed gross amounts of fatty food, blood was withdrawn, mixed with heparin and centrifuged in order to obtain the plasma. As compared to water the optical density at 620 mμ was 0.438 in human and 0.231 in dog plasma. The lipemic plasma was mixed with dye, and the resulting concentrations were 21.09 and 23.98 μg per ml in the dog and in the human plasma. The recovery from lipemic plasma, as shown by the crosses (Fig. 2), is not significantly different from that of clear plasma.

Recovery from hemolyzed blood. Clear plasma was obtained from heparinized blood. A separate 4.0 ml portion of this blood was hemolyzed with 0.1 ml of "Lorol" sulfate solution. Then, 0.5 ml of the hemolyzed blood was added along with plasma to the flask used in preparing the dye solution. Although the dye concentration was 19.67 and 19.31 μg per ml in human and in dog plasma respectively, the usual strong blue color was

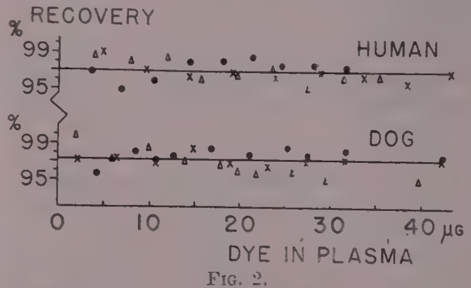

FIG. 2.

Recovery of T-1824 (corrected for impurity), 97.2% with standard deviation of 1.05% from human plasma, 97.0% with standard deviation of 1.11% from dog plasma. *Circles*, "clear" plasma; *triangles*, plasma and hemolyzed blood; *crosses*, lipemic plasma.

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completely masked. The extent of contamination was found by separately diluting the hemolyzed blood and measuring the optical density at 505 mμ(12). The hemoglobin concentration of the dye-tinged dog plasma was 0.76 g% and that of the human was 0.73 g%. The most striking thing is not so much the recovery of dye (Fig. 2, triangles) as the removal of the hemoglobin. A sharp blue band about 1 mm thick is revealed at the top of the column within a few seconds after rinsing begins. From this band some 96.8% of the dye is recovered.

Extent of separation from plasma constituents. The spectral curves of the dye in effluent obtained from the 3 types of plasma were compared with that of dye in effluent obtained by sorbing dye out of saline solution. The maximum optical density always occurred at 620 mμ. The measurements at each indicated wave length from 475 mμ to 700 mμ are in excellent agreement. Between 400 mμ and 475 mμ the dye from plasma has a somewhat greater absorption of light than the dye which was extracted from saline solution. The spectral data from human plasma, which are identical with those of dog plasma, are shown in Fig. 3. These results indicate that the extent of separation is quite remarkable. In fact this procedure can even be employed in the recovery of T-1824 from other biological fluids, such as liver and gall bladder bile.

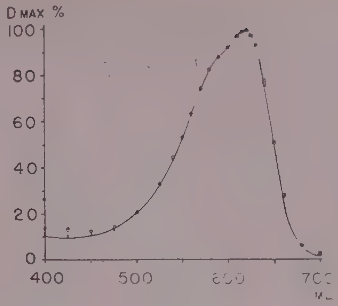

FIG. 3.

Spectral curves: relative absorption of light by T-1824 extracted from saline compared to that by T-1824 extracted from types of plasma denoted by symbols as in Fig. 2.

Summary. Consistent mean recoveries of 97% are obtained by use of a simple procedure for the stable extraction of T-1824. Dye-tinged plasma is treated with a modern soap and then quantitatively transferred to a column of paper fiber. Dye is sorbed on the paper, rinsed with saline solution and eluted with acetone:water. The various steps including the spectrophotometric measurement can be finished in one hour. Spectral curves of eluted dye show that interference from hemolysis and lipemia is removed in the rinse. This procedure improves the versatility and certainty of the dye-hematocrit method for estimation of blood volume.

The helpful advice of Professor Magnus I. Gregersen is gratefully acknowledged.

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Constriction of Thoracic Inferior Vena Cava in Rabbits Following Two-Stage Portal Ligation.* (18418)

ROGER F. MILNES.† (Introduced by George H. Stueck, Jr.)

From the Surgical Service, Veterans Administration Hospital, White River Junction, Vt., and Dartmouth Medical School, Hanover, N. H.

Rapid or gradual constriction of the thoracic inferior vena cava in animals generally produces ascites. The fluid formed by such a procedure is believed to be the result of

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† Veterans Administration Hospital, White River Junction, Vt., Mary Hitchcock Memorial Hospital, and Hitchcock Clinic, Hanover, N. H.

venous congestion in the liver. The purpose of the following experiment was to observe the effect of a previous 2-stage portal ligation on ascites produced in rabbits by thoracic inferior vena cava constriction. McKee(1,2) produced ascites in dogs within a few days by means of an adjustable aluminum band placed on the thoracic inferior vena cava midway between the heart and diaphragm. Volwiller (3) and Volwiller and co-workers(4), working with dogs, used a cellophane band placed around the thoracic segment of the inferior vena cava; the fibrous reaction of the cellophane would cause a progressive constriction of the vessel lumen to approximately one quarter of its original diameter within 3 to 4 weeks. This resulted in marked ascites. Both in animals and in man, gradual occlusion of the portal vein does not produce ascites. In monkeys, as shown recently by Milnes and Child(5), acute portal occlusion does not produce ascites. It is suggested that acute portal occlusion is tolerated by man as shown by Child(6) in his work on several patients with carcinoma of the stomach.

In the present experiment, rabbits weighing 2 to 4 kg were used. The diet consisted of stock rabbit pellets, containing 17% protein, oats and water. All surgical procedures were carried out under aseptic conditions. Postoperatively the animals were not maintained on antibiotics and no measure of intake or output was made.

Method and results. A control series of 8 rabbits was operated upon with positive endotracheal oxygen pressure and thoracotomy performed under local anesthesia. Each rabbit was subjected to constriction of the thoracic inferior vena cava, midway between the heart and diaphragm, with a polyethylene band measuring 0.5 cm in width. The vein was measured carefully with calipers so that the vessel could be reduced as nearly as possible to 60% of its normal diameter. Closure of the chest was accomplished with interrupted silk, and the lungs re-expanded by means of positive endotracheal pressure. Following operation, the presence of ascites was determined by daily paracentesis. Ascites was found to occur in 3 to 4 days. Samples of fluid were removed every 7 days for a period of 3 months and analyzed for total protein and A/G ratio. The volume that could be removed at each sampling varied from 20 cc to 40 cc, with a specific gravity range of 1.012 to 1.018. The values for total protein averaged 4.0 g per 100 cc, and the A/G ratio averaged 1.0. Over the period of 3 months, the values for total protein did not vary appreciably from the average. Interestingly, some of the animals developed edema of the posterior extremities after 4 months. No jaundice was apparent clinically in any of the rabbits. Table I outlines the 8 control experiments.

TABLE I. Ascitic Fluid in Rabbits with Thoracic Caval Constriction.

Exp.	Onset of ascites (days)	S.G.	T.P. (g/100 cc)	A/G
1	4	1.014	3.8	0.9
2	3	1.012	3.6	1.1
3	4	1.016	4.4	1.1
4	3	1.018	4.3	1.0
5	3	1.014	4.2	0.9
6	3	1.012	4.0	1.0
7	4	1.018	3.6	1.0
8	4	1.017	4.3	0.9
		Avg	4.0	1.0

Another group of 8 rabbits was operated upon and 2-stage ligation of the portal vein was performed. The procedure used to ligate the portal vein was carried out under local anesthesia in the following manner. At the first stage, the portal vein was carefully dissected free, just distal to its entrance into the liver, and a constricting band of polyethylene placed about it, causing not more than 50% constriction of the vessel's diameter. The abdomen was then closed in layers, and a period of 20 days elapsed before a secondary laparotomy was performed, at which time total ligation of the vessel was completed

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TABLE II. Ascitic Fluid in Rabbits with Thoracic Caval Constriction Following Two-Stage Portal Ligation.

Exp.	Onset of ascites (days)	S.G.	T.P. (g/100 cc)	A/G
1	3	1.012	3.6	0.9
2	4	1.013	3.5	0.8
3	4	1.016	4.2	1.0
4	4	1.016	4.0	1.1
5	3	1.015	4.4	1.0
6	4	1.015	3.9	0.9
7	4	1.013	4.1	0.9
8	3	1.014	4.0	0.9
		Avg	3.9	0.9

with silk. In none of these animals was ascites demonstrable by frequent paracentesis.

One week following the second-stage portal ligation, thoracic inferior caval constriction was performed on these rabbits, using the same technic as was used in the control series. The formation of ascites was usually demonstrated by paracentesis within 4 days. This fluid was examined for total protein and A/G ratio every 7 days for a period of 3 months. No appreciable change in average total protein or A/G ratio was noted in comparison with the animals with thoracic inferior caval constriction alone. Total protein averaged 3.9 g per 100 cc, A/G ratio 0.9, and the specific gravity varied from 1.012 to 0.016. Table II outlines the results obtained in this group of animals. These rabbits did not show clinical jaundice. There was no evidence that 2-stage ligation decreased the total volume of ascitic fluid.

Conclusion. 1. Constriction of the thoracic vena cava in rabbits produces ascites. 2. 2-stage portal ligation in rabbits does not cause demonstrable ascites. 3. In rabbits, the ascitic fluid formed by caval constriction in the chest is not affected by previous 2-stage portal ligation.

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Effects of Administration of Gonadotropic Hormone on Vitamin A Deficient Rats. (18419)

JEAN MAYER AND J. W. GODDARD.
(Introduced by F. J. Stare.)

From the Department of Nutrition, Harvard School of Public Health, Boston, Mass.

It had been shown by Mason(1), in particular, that the effect of vit. A deficiency on the male rat amounted to a virtual castration as far as secondary sexual characters are concerned. This could be interpreted as meaning that in vit. A deficiency target organs did not respond to circulating androgens or it could mean that androgens were not synthesized or released. To answer this point, Mayer and Truant(2) administered testosterone to vit. A deficient castrates and noncastrates and to their controls. It was found that the response of target organs of the deficient animals to injected androgens was essentially normal. In particular, the secondary sex organs became extremely hypertrophied even while severe losses of weight due to the deficiency were taking place. It was concluded that in vit. A deficiency, synthesis or release of androgens was interfered with. In order to find out whether this was due primarily to a testicular lesion or to a pituitary hypofunction, it was decided to study the effects of administration of gonadotropic preparations on vit. A deficient animals and their controls.

Method. The animals used were male Wistar albino weanlings, of the Harvard-Hisaw colony, 22 days of age. Their average weight was 50.75 g. These animals were kept in an air-conditioned room (about 26°C) at constant humidity and were alternately 12 hours in darkness and 12 hours in artificial light. The diet used had the following composition: Casein 25%, "Salt IV" mixture 4%, Corn

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TABLE I. Effect on Body and Organ Weight of Gonadotropic Hormone Administration.*

	Controls	Controls and hormone	Deficient	Deficient and hormone
Final† wt gain	186 g	177 g	117 g	115 g
Max. wt gain	id	id	119 g	121 g
Testes	2.65 (11.2)‡	2.30 (10.4)	1.92 (11.5)	1.46 (9.2)
Seminal vesicles§	0.39 (1.7)	1.595 (7.2)	0.33 (1.9)	1.46 (9.2)
Kidneys	2.51 (11.2)	2.65 (12.0)	1.92 (11.4)	1.92 (12.1)

* Each set of values corresponds to 7 animals. Statistical treatment is not given, as the differences observed between groups, even when small, derive their significance from the fact that they duplicate numerous classical results (*loc. cit*) obtained with testosterone.

† The difference between "Final" and "Maximum" gain weights is due to the fact that when the animals were sacrificed, the degree of deficiency reached was such that several animals had already lost weight.

‡ Figures in parentheses represent ratio: $\frac{\text{organ wt}}{\text{body wt}} \times 10^3$.

§ Prostates and epididymes showed size changes in the same direction as seminal vesicles.

oil 5%, Sucrose 65.9%, Choline 0.1%, and contained supplements of thiamine, riboflavin, calcium pantothenate, pyridoxine, biotin, folic acid, α-tocopherol, menadione (vit. K) and viosterol (vit. D). Control animals received orally 1200 I.U. vit. A every 7 days. The animals receiving hormone were injected every other day subcutaneously with 0.025 cc of "APL" chorionic gonadotropin* in saline solution corresponding to 100 international units gonadotropic activity. The other animals received control saline injections. Two weanlings were sacrificed at the start of the experimental period to provide a baseline for histological examination. Forty-four other animals were used on the experiment. They were divided into equal groups: control animals, control animals receiving hormone, deficient animals, deficient animals receiving hormone. Two animals per group were sacrificed for histological examination after 10 and 20 days respectively. All other animals were sacrificed after 30 days, by which time about one-fourth of the deficient animals were losing weight, with another fourth "plateaued." Body weights, as well as the weights of organs which have been shown to be affected by androgens(3-7) were recorded. Histological examination of the testes was conducted.

Results and discussion. Table I gives the final and maximal weight gains of all groups as well as the weights of kidneys, testes and seminal vesicles, and the ratios of the weights of these organs to body weight. In the control animals, the classical effects of androgen administration can be noted. Body and testes weight are slightly decreased, kidney weight is slightly increased. The considerable hypertrophy of the secondary sex organs—seminal vesicles as well as prostates and epididymes—obtainable by testosterone injection is, as could be expected, duplicated by gonadotropic hormone administration. In the deficient animals the weight response of testes, kidneys and especially seminal vesicles follows that of the controls. From consideration of organic weights alone no qualitative or, (when the differences in body weight are taken into consideration) quantitative differences appear between the reactions to gonadotropic hormone of controls and deficient animals. This can be taken to demonstrate that the primary "castrating" lesion in vitamin A deficiency is the decrease of the secretion of pituitary gonadotropic hormone rather than the inability of the testes to produce androgens upon gonadotropic stimulation.

Histological examinations proved fruitful.

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* We wish to thank Ayerst, McKenna and Harrison, Ltd. for their generous gift of gonadotropic hormones.

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As classically described administration of gonadotropic hormone had little detectable effect on seminiferous tubules and spermatogenesis in normal animals. It had a striking effect on the interstitial tissues, more than doubling the number of Leydig cells. Vitamin A deficiency alone, as previously indicated(1,8) decreases the number of spermatozoa and spermatides. The number of first and second order spermatocytes was normal, with the spermatogonias somewhat smaller and generally quiescent. The picture in simple vitamin deficiency is therefore one of decreased spermatogenesis, but, at least in the relatively early stage, without radical, generalized pathology. On the other hand, when gonadotropic hormone was administered to vitamin A deficient animals, very definite lesions were observed. Striking increases in the number of Leydig cells (interstitial tissue) were obtained as in normal animals, but many cells appeared to be in a state of lysis and presented abnormally shaped nuclei. Delicate interconnective tissue fibrils could be observed between many cells (early collapse fibrosis). While gonadotropic hormone administration had practically no effect on spermatogenesis in the normal animal, it had definitely inhibitory effects in the deficient animal. No ripe spermatozoa were visible. Spermatides were also totally absent. The number of spermatocytes of the first and second order was decreased, but many of them presented mitotic figures; the "block" appeared therefore to be between the second order spermatocyte and the spermatide stages. At the level of the spermatocytes many agglutinated and partially lyzed cells with vacuoles were to be seen. Spermatogonias, although somewhat fewer and smaller than normal, presented many mitotic figures, thus confirming the localization of the block, and indicating that a new series of spermatozoa production would be possible if vitamin A became again available. The number of Sertoli cells was increased. Administration of gonadotropic hormone to vitamin A deficient animals, while it must still elicit production of androgens by the interstitial tissue, thus also results in effects not only quantitatively but qualitatively different from those produced in normal animals. Decrease in pituitary gonadotropic activity in vitamin A deficiency could thus perhaps be considered one of the defense mechanisms of an organism which lacks an essential tissue nutrient. Differential effects of follicle stimulating (FSH) and luteinizing (LH) hormones are being studied.

Summary. 1. To find out whether degeneration of secondary sex organs in vit. A deficient male rats is due to the inability of the testes to produce androgens or to a decrease in the activity of the pituitary, gonadotropic hormone was administered to vit. A deficient animals and to their controls and the effects compared with control conditions. 2. The weight response of "target organs," *e.g.* kidneys and seminal vesicles was found to be essentially normal in vit. A deficient animals, thus showing that androgens could still be produced by the testes. 3. On the other hand, it was observed that gonadotropic hormone had a definitely injurious effect on vit. A deficient animals, in particular producing a block at the spermatocyte level with consequent arrest of spermatide production.

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Effect of Furacin (5-nitro-2-furaldehyde semicarbazone) on Various Sulfhydryl and Non-sulfhydryl Enzymes.*† (18420)

Morris N. Green, Edward C. Heath, and Irving Yall.
(Introduced by Albert G. Hogan.)

From the Department of Bacteriology, School of Medicine, University of Missouri, Columbia.

Furacin or 5-nitro-2-furaldehyde semicarbazone inhibits the growth of a variety of gram positive and gram negative bacteria(1) and the oxidation of glucose and pyruvate in bacteria(2). In addition to Furacin, other 5-nitro-furans inhibited the oxidation of glucose(3) and pyruvate(4) by bacteria while the corresponding non-nitro analogues were completely inactive(3,4). The growth of a transplanted fibrosarcoma in mice(5,6) was also inhibited. In the following experiments the action of Furacin on various sulfhydryl and non-sulfhydryl dehydrogenases and hydrolysing enzymes from bacteria and tissues will be described.

Experimental. Experiments with isolated enzyme systems. In these experiments the effect of Furacin on purified urease (Bios), crystalline pepsin (Armour), crystalline trypsin (Armour) and papain (Nutritional Biochemicals) was observed. Urease activity was determined by the method of Folin and Wu(7) using Nesslerization. The sulfhydryl character of the enzyme has been studied by Hellerman and his group(8). The urease was exposed to 3.6 x 10^{-4} M (1:15,000) Furacin in a water bath at 37°C for periods varying from 15 minutes to an hour, along with a control containing water, both at a pH of 7.4. At the end of the exposure period, the urea substrate, buffered with Folin pyrophosphate buffer, pH 7.6, was allowed to react with the substrate for 15 minutes after which the reaction was stopped with normal hydrochloric acid. Urease activity was determined in 5 ml portions of solution containing 0.6 mg of urease and 0.4 mg of urea in all experiments. Reactivation experiments were performed with neutralised cysteine hydrochloride (Nutritional Biochemicals) using exactly the same amounts of enzyme, substrate and Furacin as above. The molar concentration of cysteine was 100 times that of Furacin. The results of these experiments are given in Table I. The observed inhibition of urease activity depends on the duration of exposure to Furacin. Reactivation with cysteine varied in the same manner. Exposure to the non-nitrated analogue of Furacin, 2-furaldehyde semicarbazone, 2.14 x 10^{-3} M (1:2,500) gave no detectable inhibition.

TABLE I. Effect of Furacin on Activity of Jack Bean Urease and Its Reactivation by Cysteine.

Initial exposure time to furacin, min.	Molarity of furacin	Mg of ammonia N per 5 ml sol.		% inhibition	% reactivation with cysteine 3.6 × 10^{-2} M
		Without cysteine	With cysteine		
15	3.6 × 10^{-4}	.046	.060	29.0	72.5
15	—	.065	.064		
30	3.6 × 10^{-4}	.030	.051	50.0	70.0
30	—	.060	.058		
60	3.6 × 10^{-4}	.023	.040	56.6	56.7
60	—	.053	.054		

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† The authors wish to thank Dr. L. A. Sweet of Parke, Davis and Co. for the arsenicals and Drs. W. L. Stillman and L. Eugene Daily of the Eaton Laboratories for the furan derivatives used in this study.

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No inhibition was noted for pepsin or trypsin after an exposure to 1:10,000 Furacin for one hour, or for papain after an exposure of 2 hours to 1:7,500 Furacin. Pepsin and trypsin are classified as non-sulfhydryl enzymes (9) while Fruton and Bergmann(10) and Jaffe(11) obtained results which places papain in a controversial category. The formol titration method of Brown(12) was used in assaying the activity of these enzymes. The glass electrode was used instead of indicators for determining the end point of the formol titration.

Experiments with bacterial and tissue dehydrogenases. Using the Thunberg technic, the effect of Furacin on the formic, malic, and succinic dehydrogenases of *E. coli* was determined(2). In accordance with Gale's(13) experiments, the formic dehydrogenase of *E. coli.* is presumably non-sulfhydryl in character. This was also indicated by our experiments, since pCMB (*p*-Chloromercuribenzoate) and Mapharsen (3-amino-4-hydroxyphenyl-arsineoxide hydrochloride) in concentrations of 1.8 x 10^{-5} M and 1.3 x 10^{-4} M respectively do not affect this dehydrogenase while the malic and succinic dehydrogenases are inhibited at these levels. The α-glycerophosphate dehydrogenase of rabbit thigh muscle is non-sulfhydryl according to Hopkins and Morgan(14). No change in the reduction time of methylene blue with this enzyme from rabbit muscle was observed using Furacin (5.4 x 10^{-4} M).

The malic, lactic, formic and α-glycerophosphate dehydrogenases of *E. coli* were also studied aerobically in the Warburg respirometer. The method of Barron and Singer(15) was used for the malic dehydrogenase while a method similar to that used by Green(2) was used for the formic dehydrogenase. During a 2 hour period of observation, no inhibition of oxygen uptake was observed with the formic dehydrogenase in the presence of 3.6 x 10^{-4} M Furacin while the other dehydrogenases were inhibited. Upon the addition of 100 moles of cysteine per mole of Furacin, complete reversal of the malic inhibition was obtained while no effect was observed upon the formic dehydrogenase. The non-nitro analogue of Furacin, 2-furaldehyde semicarbazone, in a concentration of 1.4 x 10^{-3} M gave no detectable inhibition of the malic and formic dehydrogenases. Semicarbazide was used as a fixative in experiments with the malic and lactic dehydrogenases.

Experiments with organisms susceptible and resistant to Furacin. *E. coli* and *S. aureus* were rendered resistant to Furacin by transplantation in increasing amounts of the drug. The control organisms were carried through a similar series of transfers in the same media without Furacin. Several of the previously discussed enzyme systems were tested with the parent susceptible and resistant strains in the presence of Furacin and other sulfhydryl agents. The results of these experiments on some of the dehydrogenases previously mentioned using *E. coli* show that Furacin resistant organisms appear to have developed some cross resistance to sulfhydryl agents especially pCMB and Mapharsen. The other arsenicals (p-aminophenylarsineoxide, p-carboxyphenylarsineoxide) did not show this effect in all cases. The resistant strain of *E. coli* could not be differentiated from the susceptible strain by the usual cultural reactions.

In a series of experiments, the amount of Furacin and sulfhydryl inhibitor was kept at a constant level, while the weight of organisms varied from 0.75 mg to 1.5 mg per test. At the lower levels (0.75 mg) the resistant strain was relatively insensitive to Furacin and pCMB, while the susceptible strain was relatively sensitive. When both resistant and susceptible strains were increased to a higher level (1.5 mg) both strains became relatively

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TABLE II. Effect of Furacin on Various Dehydrogenases.

		Methylene blue method		O_2 uptake method	
Enzyme source	Substrate	Conc. furacin, M	Minutes for 90% reduction	Conc. furacin, M	μl O_2 uptake in 60 min.
E. coli	Malate	5.4×10^{-4}	21	3.6×10^{-4}	32
,,	,,	—	7	—	102
,,	Succinate	5.4×10^{-4}	20		
,,	,,	—	8		
,,	Lactate			3.6×10^{-4}	237
,,	,,			—	514
,,	Formate	5.4×10^{-4}	6	3.6×10^{-4}	129
,,	,,	—	6	—	133
,,	α-glycerophosphate			3.6×10^{-4}	46
,,	α-glycerophosphate			—	125
Rabbit thigh muscle	α-glycerophosphate	5.4×10^{-4}	15		
,, ,, ,,	α-glycerophosphate	—	15		

TABLE III. Effect of *p*-Chloromercuribenzoate on Malic Dehydrogenase of a Strain of *E. coli* Susceptible and Resistant to Furacin.*

		Minutes for 90% reduction							
		0.75 mg org./test		1.0 mg org./test		1.25 mg org./test		1.5 mg org./test	
Inhibitor	Molar conc.	R†	S†	R	S	R	S	R	S
p-Chloromercuribenzoate	3.6×10^{-5}	α‡	α	31	α	6	23	4	11
,,	1.8×10^{-5}	16	38	7	16	6	12	4	6
,,	9.0×10^{-6}	12	21	7	9	6	7	4	6
Furacin	5.4×10^{-4}	12	50	7	21	6	16	4	10
Control	—	12	16	7	8	6	7	4	5

* Each test was performed in a total vol. of 1.25 ml containing phosphate buffer, pH 7.2, 0.1 ml; sodium malate 0.5 M, 0.1 ml; semicarbazide 1.0 M, 0.1 ml; organisms as above 0.25 ml; methylene blue 0.001 M, 0.1 ml; distilled water or inhibitor, 0.6 ml.

† The symbols R and S represent the resistant and susceptible strains of the organism respectively.

‡ α = decolorization time greater than 1 hr.

insensitive to Furacin and pCMB. These results are given in Table III.

Urease from susceptible and resistant strains of *S. aureus* grown in 2% urea medium was tested at various levels ranging from 2.0 mg to 14.0 mg of organisms per test. Inhibition was noted by Furacin and pCMB in the susceptible strain in all cases, while the resistant strain was insensitive to both of these reagents.

Discussion. It appears from these results that Furacin inhibits some sulfhydryl enzymes. Not all sulfhydryl enzymes seem to be inhibited since Furacin had no effect on papain. However there is some controversy as to the sulfhydryl nature of this enzyme(10,11). An indiscriminate inhibition of all sulfhydryl enzymes may result in acute toxicity and may not be characteristic of a selective chemotherapeutic agent. The exact nature of sulfhydryl inhibition with Furacin remains to be determined by further experiments.

Furacin does not appear to act as an indiscriminate dehydrogenase inhibitor. The formic dehydrogenase of *E. coli* and the α-glycerophosphate dehydrogenase of rabbit thigh muscle which presumably are non-sulfhydryl in character are not inhibited by Furacin.

The experiments with Furacin susceptible and resistant strains of bacteria suggest several possible explanations. It is conceivable that in the process of the development of resistance, the overall sulfhydryl character of the bacterial dehydrogenases may be altered. Another possibility may be a change in the permeability of the cell or the emergence of a divergent metabolic pathway. This problem can be clarified by isolating and comparing the properties of the same sulfhydryl enzymes from the parent susceptible and resistant strains.

Summary. Furacin inhibits purified jack bean meal urease as well as urease from a susceptible strain of *S. aureus*. Pepsin, trypsin and papain are not inhibited. The malic, succinic, lactic and α-glycerophosphate dehydrogenases of a susceptible strain of *E. coli* are inhibited by Furacin while the formic dehydrogenase is not inhibited. The α-glycerophosphate dehydrogenase of rabbit thigh muscle is not inhibited. Several dehydrogenases from a strain of *E. coli* resistant to Furacin show some cross resistance to p-chloromercuribenzoate and some trivalent arsenicals. A similar phenomenon was observed with the urease from a strain of *S. aureus* resistant to Furacin. It is concluded that Furacin inhibits some enzymes requiring active sulfhydryl groups.

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Right and Left Atrial Pressures in Successive Stages of Oligemic and Normovolemic Shock.* (18421)

HOWARD F. VAN NOATE.† (Introduced by Carl J. Wiggers.)
From the Department of Physiology, Western Reserve University School of Medicine, Cleveland, O.

Using dogs under morphine and barbital anesthesia, with their chests open and under controlled lung inflation, C. J. Wiggers(1) studied the circulatory failure which developed as a result of prolonged hemorrhagic hypotension, and again after reinfusion of the withdrawn blood. It was found that the experiments could be divided into two approximately equal groups: (1) Those in which the circulatory failure developed after reinfusion as right atrial pressure declined, and (2) those in which it supervened despite the fact that right atrial pressure was maintained at or above control levels. The question arises whether the reduction in cardiac output—mainly responsible for the decline of arterial pressure after transfusion(2,3)—is accompanied by changes in left atrial pressure which are in accord or at variance with those in the right atrium. It was the purpose of this investigation to study these pressure relations by simultaneous registration of right and left atrial pressures, for it has never been proved that the beats of the two ventricles are coordinated so perfectly during oligemic and normovolemic shock that changes in filling pressure for the right ventricle are automatically accompanied by similar quantitative or even directional changes on the left side.

Methods. Dogs weighing from 10 to 15 kilos were anesthetized with morphine and sodium barbital at least 3 hours prior to any observations. The chest was opened in the midline, utilizing an electric cautery to minimize blood loss. The trachea was cannulated and respirations were maintained by means of an artificial respirator. Optical tracings of the right and left atrial and the central aortic pressures were taken on a rapidly moving photokymograph by means of calibrated Gregg manometers of adequate frequency and appropriate sensitivity. These were connected by lead tubing to rigid cannulae introduced respectively through the right jugular vein, a left pulmonary vein and the subclavian artery. A segment capsule in the air-

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* Supported by a grant from the American Heart Association for the purpose of training cardiovascular investigators.

† Postdoctorate Research Fellow, National Heart Institute, U. S. Public Health Service.

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line from the artificial respirator permitted the optical recording of the respiratory cycle. In addition, mean pressure was recorded continuously on a smoked drum to facilitate standardization of the hypotension levels. A femoral artery and vein were cannulated to withdraw and reinfuse blood. Hemorrhagic shock was produced according to the method developed in this laboratory(4,5) except that a stepwise type of bleeding suggested by Lawson(6) was employed to lower arterial pressure to the 50 mm Hg level within 30-45 minutes. Optical records were taken at frequent intervals and each was calibrated with respect to its individual base line. All records were compared at the same phase of lung inflation. This, in addition to the fact that the respiratory depth and rate were maintained constant, eliminated changes in atrial pressures due to cyclic respiratory changes in venous return, pulmonary resistance or hydrostatic pressure. Of the various ways for determining atrial pressures on optical records(5), measurement of the instantaneous pressure at the end of systole (V pressure) was found to be most expedient and reliable for comparison of records taken at wide intervals of time. It may be recalled that such easily determined pressures represent the pressure initially available for filling of the respective ventricles after the A-V valves open. Whenever clearly indicated, the pressure at the onset of ventricular systole, the Z pressure, was also measured. This theoretically corresponds to the initial intraventricular tension under which ventricular contraction starts.

Results. Shock consistently followed use of the bleeding and transfusion techniques in 16 dogs, but the purpose of this study could only be fulfilled when complete sets of reliable records were realized at spaced intervals throughout the entire 7 to 9 hours required for each experiment. This *sine qua non* proved difficult. Many experiments yielded only partial information or needed to be wholly discarded owing to technical difficulties involved. Among these were experiments in which (a) atrial pressure curves (particularly the left) were obviously distorted, (b) the position of the atrial sounds required readjustment as the volumes of the cardiac chambers changed, (c) the depth and rate of lung inflation could not be kept constant, (d) the animal's temperature could not be maintained at an even control level, or (e) additional anesthesia was required.

As a result of these vicissitudes of experimentation only six experiments were realized in which conditions were wholly satisfactory. Of these, only one experiment the data of which are plotted in Figure 1 displayed a

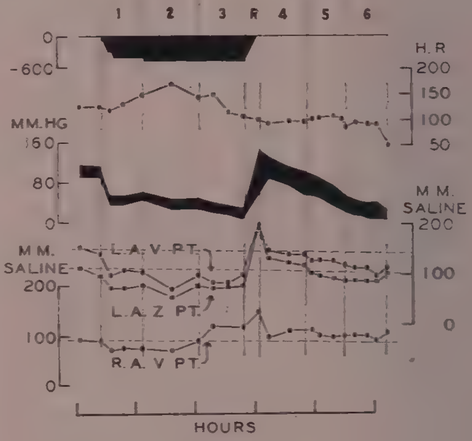

FIG. 1.

Graph of data showing sequence of some cardiodynamic events following bleeding to hypotensive levels and subsequent reinfusion of blood, as indicated in cc by upper black area. Vertical lines indicate (approximately) successive phases of shock adapted to recent nomenclature adopted by Wiggers(5). Order of curves: bleeding and reinfusion volumes; heart rate (H.R.); arterial blood pressure; V and Z points of left atrial pressure (L.A.V. PT. and L.A.Z. PT); V point of right atrial pressure (R.A.V. PT). All atrial pressures are related to level of animal board as zero and show relative changes only; all values are 55 to 60 mm saline higher than actual atrial pressure. Right atrial Z pressures followed V pressures so closely that their plot is omitted to prevent confusion in graphing. *Phases*: 1-3, oligemic shock; 1, bleeding and simple hypotension; 2, impending stage; 3, critical stage; R, reinfusion; 4-6, normovolemic shock; 4, compensatory stage; 5, progressive stage; 6, terminal stage.

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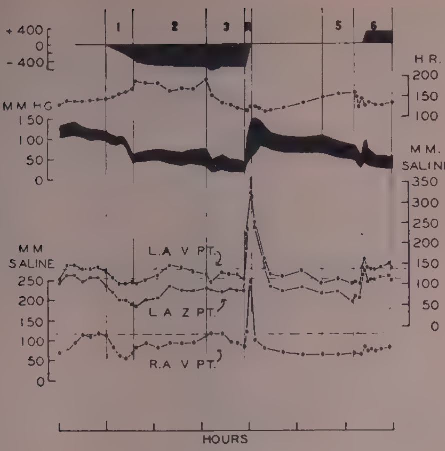

FIG. 2.
Same as Fig. 1.

sustained elevation of right atrial pressure similar to that of a group reported by C. J. Wiggers(1) during the critical period of oligemic shock(3) and during the circulatory failure following transfusion(5,6). A survey of left atrial pressures in this experiment reveals that (a) during the critical phase(3) both Z and V pressures declined in the left atrium, (b) during the period of reinfusion (R) left atrial V and Z pressures rose much more than corresponding right atrial pressures, and (c) during the circulatory failure following transfusion(5,6), V pressure in the left atrium fell below control values, while it remained elevated in the right. The Z pressures in the left atrium also declined but not to levels below control values until the terminal stage(6).

While more experiments are needed, this single experiment supported by casual data from other incomplete experiments, suggests that elevation of right atrial pressure found in a fair proportion of Wiggers' experiments (1) may not be accompanied by a maintenance of left atrial pressure.

In 2 experiments, an example of which is shown in the plot of Figure 2, V pressures fell in both atria during the bleeding(1). Both tended to return to control levels during the impending and critical phases of oligemic shock(2,3). In three experiments, right and left atrial V and Z pressures were reduced during hemorrhage and failed to rise during the subsequent impending and critical periods.

Following reinfusion the elevation of left atrial V and Z pressures greatly exceeded those in the right atrium in all 5 cases. In the right atrium this rise was followed by a prompt drop in pressures to subnormal levels in all 5 experiments. In 3 of these cases, the decline of left atrial pressure stabilized at or above control levels; in the other 2 it fell below them. In the experiment plotted in Fig. 2, an additional 320 cc of crossmatched blood was administered during the terminal stage. This resulted in a slight elevation of right atrial and a marked rise of left atrial pressures to control levels. Despite this rise, systolic, diastolic, and pulse pressures continued to diminish until the end.

Since changes in heart rate have a determining effect on atrial pressures the experimental data were reviewed in an effort to find times in different stages of the experiment when the heart rate remained relatively constant. Two comparisons of left and right atrial Z pressures under such conditions are recorded in Table I.

The data of Exp. 12 were obtained from one of the 2 animals in which both right and left atrial pressures decreased below control values after reinfusion. It will be noted that left atrial Z pressure decreased to a greater extent than the right, both during the stage of impending oligemic and the compensatory and terminal stages of normovolemic shock.

The data of Exp. 10 were obtained from one of the 3 animals in which right atrial Z pressure decreased, while left atrial Z pressure was sustained during postinfusion phases. During the stage of critical oligemic shock both right and left atrial Z pressures decreased very little, but during the progressive stage of normovolemic shock right atrial pressure fell while that of the left atrium remained near a control level.

Such comparisons indicate that the alterations in atrial pressures are not dominated by the changes in heart rate but are due to other causes.

Interpretation of Atrial Pressure Changes. Briefly recapitulated, 3 patterns of change in

TABLE I.

Exp.	Phase	Heart rate per min.	Pulse pressure, mm/Hg	Mean blood pressure, mm Hg	Right atrial Z pressure, mm H_2O	Left atrial Z pressure, mm H_2O	Cardiac index by pulse contour method
12	Control	174	28	113	105	105	3.04
	Impending oligemic stage	175	16	47	80	65	1.59
	Compensatory normovolemic stage	174	43	93	95	70	3.53
	Terminal normovolemic stage	178	23	36	70	60	1.53
10	Control	117	49	116	135	130	1.94
	Critical oligemic stage	132	18	24	130	130	1.08
	Compensatory normovolemic stage	140	55	64	135	130	2.44
	Progressive normovolemic stage	130	19	29	80	125	1.22

the atrial pressures were observed in the post-infusion shock phase: (a) a decrease in both the right and left atrial pressures to low levels, the left decreasing to a greater extent, (b) a decrease in the right atrial pressure to a low level, but a failure of the left to decrease below a control level, and (c) a decrease in the left atrial pressure, the right remaining normal or elevated. If we assume that the post-transfusion circulatory failure in these experiments had been due solely to reduction in venous return(3), right atrial pressure would be expected to decline. With the further probable assumption that the left heart receives a proportional decrement of flow, left atrial pressures should decline even more at the Z and V points[‡](7,8). Such a combination of effects was found in 2 experiments only. In the 3 records in which right atrial pressures decreased below control values in the post-infusion phase of circulatory failure, but in which the left did not decrease to the expected amount or actually remained at or above control values, the fall in right atrial pressure would also indicate a decreased venous return. The failure of the left atrial pressure to fall to an even greater extent, as would be expected, however, strongly suggests a depression of the left ventricular muscle in these cases. In the sixth animal, the right atrial pressure remained elevated while the left atrial pressure decreased in the post-infusion phase. Since the initial hemorrhage and the reinfusion indicated a greater distensibility of the right atrium than of the left, some other factor must have operated. The elevation of V and Z pressures in the right atrium allows the conclusion that pressure for filling the right ventricle was adequate. The decline of left atrial V and Z pressure strongly suggests that less blood is returned through pulmonary veins to the left atrium. Excluding a highly improbable increase in pulmonary resistance great enough to impede blood flow to the left atrium, one is forced to the conclusion that depression of the right myocardium must have been concerned. Evidence of a dominant left ventricular depression seemed to be present in 3 of these experiments and signs of right ventricular depression in one of them.

[‡] This difference is consonant with an elementary proposition emphasized by Little, *et al.*(7,8) that owing to the lesser distensibility of the left atrium the pressure increment required to press a certain additional volume into the left atrium must be greater than that required to force the same additional volume into the right atrium. Obviously, the lesser distensibility of the left atrium *per se* does not create the additional necessary pressure, as wrongly inferred by Nahar *et. al.* (PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 737).

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Summary and Conclusions. 1. Simultaneous changes in right and left atrial pressures together with aortic pressure pulses were successfully recorded through the experiments in 6 out of 16 dogs which were submitted to standardized procedures of bleeding and reinfusion developed in this laboratory. 2. An analysis of the results supports previous observations from this laboratory that myocardial depression can supervene during the circulatory failure which develops after prolonged hemorrhagic hypotension and subsequent reinfusion of the withdrawn blood. It extends these observations in showing that such myocardial failure may affect either the left or right ventricle dominantly.

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Lysozyme Production in Response to Injury of Gastrointestinal Tract in Dogs. (18422)

HUGO C. MOELLER,* HOMER C. MARSHALL AND JOSEPH B. KIRSNER.
(Introduced by Walter L. Palmer.)
From the Frank Billings Medical Clinic, Department of Medicine, University of Chicago.

The recent investigation of Meyer and his co-workers suggesting a relationship between lysozyme(1) and certain ulcerative diseases of the alimentary tract has aroused widespread interest(2,3). The evidence suggested that lysozyme locally initiated the lesions of chronic ulcerative colitis by removal of the surface mucus, with dissolution of the mucous cells; necrosis of the denuded tissue by proteolytic enzymes, presumably furnished by micro-organisms, subsequently ensued. Wang, Grossman, *et al.*(4) showed that lysozyme produced erosions and hemorrhages in the gastric and colonic mucosa of rats, and increased the digestive effect of hydrochloric acid and pepsin *in vivo*. Lysozyme was considered capable of damaging mucous membranes, but no evidence was adduced that it removes surface mucus from the mucosa.

The hypothesis that the polysaccharolytic activity of lysozyme leads to gastrointestinal ulceration has not been substantiated, as yet, by the demonstration of a lysozyme substrate in the gastrointestinal wall or lumen(5,6). High lysozyme titers have been demonstrated in healing skin incisions, callus of healing rabbit fractures, and in inflammatory exudates(7, 8,9). The possibility exists, therefore, that lysozyme is a result rather than a cause of the inflammatory reaction in chronic ulcerative colitis. To evaluate this possibility, the concentrations of lysozyme were measured before and during experimentally induced injury to the bowel in dogs.

Methods of Study. Lysozyme titers were determined by a modification of Fleming's turbidimetric method(10). Repeated determinations of crystallized egg white lysozyme indicated an end point at 0.001 mg lysozyme per cc of material. The test is gross and does not detect small amounts of lysozyme or minor changes in concentration; it has the advantages of simplicity and rapidity, facilitating the simultaneous examination of a large number of specimens.

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* Public Health Service Research Fellow of the National Institutes of Health.

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In a long-term study of the effect of prolonged hypermotility upon the gastrointestinal tract(11), in progress in our laboratory since 1948, adult mongrel dogs with a normal rectal mucosa, as seen at proctoscopy, received daily intramuscular injections of Mecholyl chloride† suspended in a beeswax-peanut oil mixture. Fecal specimens from some of these animals were tested for lysozyme before and after the administration of the Mecholyl mixture. Lysozyme is not demonstrable in the feces of normal dogs by the method used. Mecholyl in doses of 2 mg/kilo/24 hours produced bloody diarrhea with variable side effects, consisting chiefly of lacrimation, salivation, and occasionally vomiting. Proctoscopy revealed an edematous, friable, hyperemic, bleeding mucosa; this state could be made to persist for many months by continued injections. Autopsy revealed intense hyperemia and superficial erosions of the mucosa in the stomach, small intestine, and colon. Microscopic examination demonstrated epithelial necrosis, distention of mucosal capillaries, extensive intramucosal hemorrhage, and edema of the submucosa. Similar changes have been described by Wener and Hoff(12). Irregular elevations of the lysozyme titers in the feces of these animals were demonstrable after the administration of Mecholyl.

To quantitate and better interpret results, blind ileocolic pouches were established surgically in 6 normal dogs. One month after surgery the pouch contents were tested for lysozyme before and after stimulation with Mecholyl (Fig. 1). Thirty determinations on a 1:1 dilution of secretions from blind pouches before treatment revealed no lysozyme activity. In contrast, more than 40 determinations on the pouch secretions in the same animals at intervals of 1 to 20 days of daily Mecholyl administration revealed lysozyme activity ranging up to dilutions of 1:1000.

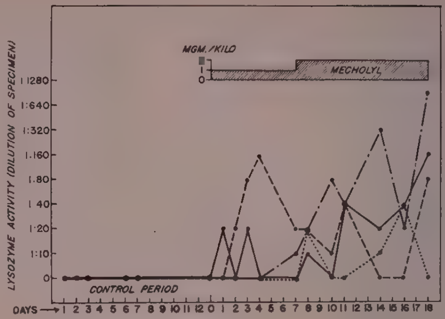

FIG. 1.

Output of lysozyme in colonic pouches of dogs before and during administration of mecholyl.

Six dogs under intravenous nembutal anesthesia were subjected to cauterization of the rectum by an electrocautery introduced through the proctoscope. Washings of the cauterized bowel were tested for lysozyme at varying intervals thereafter. Uniformly high concentrations of lysozyme were found in more than 30 determinations. At times, 20 cc of washing material from the 15 cm section of bowel contained appreciable lysozyme activity when diluted 1:5000 with saline. These concentrations are higher than those usually encountered in the feces or colonic mucus from patients with active, severe chronic ulcerative colitis (Fig. 2).

To determine whether or not the lysozyme is produced locally at the site of injury, 2 blind loops of colon were established. One of these loops was cauterized and the other served as a control. Lysozyme titers of saline washings of the 2 loops were compared at intervals for 24 hours. Lysozyme was found in both loops 2 hours after the operation. The lysozyme remained at a low level for the

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† Furnished through the generosity of Merck and Co., Rahway, N. J.

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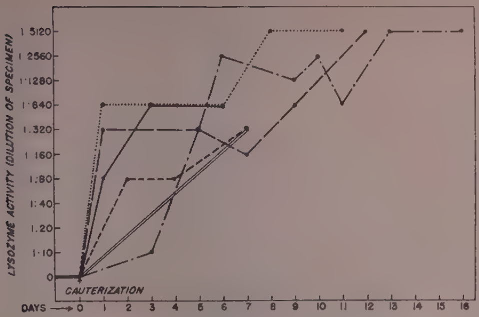

FIG. 2.

Output of lysozyme in rectum of dogs before and after cauterization.

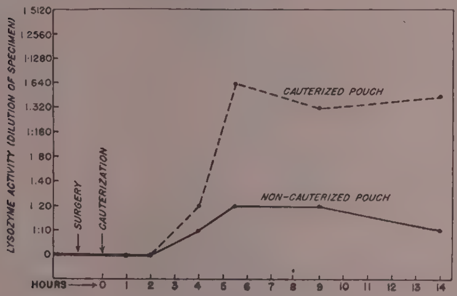

FIG. 3.

Early lysozyme activity of injured bowel.

duration of the experiment in the non-cauterized loop. Lysozyme activity of the washing from the cauterized loop continued to rise and reached a peak within 5½ hours after surgery and cautery (Fig. 3). Tissue lysozyme was detected at 1:20 dilution in a saline homogenate of the non-cauterized loop and in a 1:640 dilution of the cauterized loop homogenate. Both specimens were taken 15 hours after surgery.

Comment. The marked rise in production of lysozyme by the canine gastrointestinal tract injured by Mecholyl stimulation or by electrocautery suggests that the observed increase in lysozyme in the feces and bowel of patients with chronic ulcerative colitis may be a result of the disease process. Lysozyme seems to be produced locally in injured bowel. The marked increase in lysozyme production occurring within 5½ hours after cautery suggests that its increase is part of an early inflammatory process. Its precise biological significance remains to be determined.

Summary. 1. Lysozyme is produced in moderately large amounts by the gastrointestinal tract of dogs subjected to chronic stimulation with Mecholyl. The increase in lysozyme is demonstrable in the bloody diarrheal stools of intact animals and in material aspirated from isolated pouches of colon. 2. Cautery of canine rectum stimulates the local production of large amounts of lysozyme within 5½ hours after injury. 3. The rapid production of lysozyme by injured bowel suggests that it is an early accompaniment of the inflammatory reaction and that its presence is a reflection rather than a cause of ulcerative alimentary disease.

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Susceptibility to Estrogen of Breast, Vagina, and Endometrium of Various Strains of Mice.* (18423)

MARTIN SILBERBERG AND RUTH SILBERBERG.

From the Snodgras Laboratory, City Hospital Division, Saint Louis, Mo.

Strain differences in the susceptibility to breast cancer have been attributed by Loeb to variations in the sensitivity of the mammary gland to estrogen(1,2). According to other investigators, ovaries of mice of different strains produce different amounts of estrogen, and the larger the output of estrogen, the more readily mammary cancer will develop. Studies of vaginal smears and of whole mounts of breasts have indicated that the responses of mammary gland and vagina do not run parallel within the same animal

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* This investigation was supported by the National Cancer Institute, National Institutes of Health.

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(3-7). The following investigation represents another attempt to further analyze this question.

Material and methods. Forty-two female mice—14 each of the closely inbred strains C57 black, Dba, and A—were used. The animals were raised in our laboratory and maintained on a standard diet of Purina Laboratory Chow and water available at all times. At the age of 3 to 4 weeks, the ovaries were removed, and 2 weeks later subcutaneous injections of alpha estradiol benzoate[†] dissolved in sesame oil were begun. The mice received 0.03 mg of the hormone once weekly for 1 or 2 weeks, 1, 2, 3, 5, or 8 months. At the end of these periods, 2 animals of each strain were killed. Of each animal 4 mammary glands, uterus and vagina were removed. All 4 mammary glands as well as uterus and vagina were cut in semiserial sections and studied microscopically. The findings were graded, as previously described in detail(8).

Microscopic Examination. Mammary gland. Resting state: The ducts are scanty, narrow, and lined by low cuboidal epithelium. Stimulation grade I: The ducts are enlarged, elongated and contain secretions; there is papillary infolding and outbudding of ductal epithelium with some mitoses. Stimulation grade II: The ducts are markedly hypertrophic and hyperplastic with numerous mitoses in the epithelium; abundant secretions and alveolar growth are present.

Vagina. Resting state: There are 4 to 6 rows of keratinizing epithelial cells, the basal layer showing 1 mitosis in about 500 cells. Stimulation grade I: The epithelium is conspicuously keratinized and hyperplastic, and is composed of up to 12 layers of cells with 1 mitosis in about 200 basal cells. Stimulation grade II: The epithelium has undergone further keratinization and hyperplasia, and 15 or more layers of cells were counted with 1 mitosis in about 100 basal cells.

Endometrium. Resting state: The glands are small and do not secrete. Stimulation grade I: The glands are enlarged and contain secretion; they are lined by columnar epithelium that shows mitotic proliferation; there is early epidermization of the uterine glands closest to the muco-epidermal junction. Stimulation grade II: The glands are tortuous, cystically dilated and densely packed; they are lined by hyperplastic epithelium with abundant mitoses, and often contain secretions. Epidermization of the glandular epithelium is marked.

Results. The findings are summarized in Table I.

Mammary gland. In mice of strain C57 treated with estrogenic hormone for one or 2 weeks, the ducts were resting or at best slightly dilated. In mice of strain Dba, there was growth stimulation of grade I after one, and stimulation of grade II after 2 weeks' treatment. In mice of strain A, alveolar growth was distinct already after one injection of the hormone. After one month of treatment, growth stimulation was still slight in mice of strain C57 (Fig. 1), while alveolar growth was accentuated in mice of strain Dba (Fig. 2), and numerous alveolar nodules had been formed in mice of strain A (Fig. 3). In mice of strain C57, ductal growth was marked after 2 months of observation, and only after 3 months alveolar growth was noted. After 5 or 8 months, alveolar growth was widespread in a few mammary glands of mice of strain C57; in corresponding animals of strains Dba and A, multicentric alveolar growth and adenomatous nodules were present; the epithelium showed hyperchromatic or pycnotic nuclei, and in 2 mice mammary cancer had developed.

Vagina and endocervix. In mice of strain C57, the growth stimulation was of grade II as early as after one injection of the hormone: There was abundant keratinization of the epithelium, which still increased during the later stages of the experiment. In mice of

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TABLE I. Microscopic Findings in Mammary Glands, Vagina, and Endometrium in the Various Strains Following Treatment with Estrogen.

		Mammary gland				
Strain	Estrogen inj. for	No. ducts in one low power field: 48 mm obj., 10× ocul.	Ductal growth*	Alveolar growth†	Vagina‡	Endometrium§
C57 Black	1 week	6-12	(+)	0	++	++
	2 "	9-16	(+)	0	++	++
	1 month	7-15	(+)	0	++	+++
	2 "	12-24	+	0	++	+++
	3 "	14-35	++	+	++	+++
	5 "	16-38	++	+	++	+++
	8 "	14-40	++	++	++	+++
Dba	1 week	8-16	+	0	+	+
	2 "	8-26	++	+	+	+
	1 month	24-40	++	+	++	++
	2 "	25-35	++	++	++	+++
	3 "	22-35	++	++	++	+++
	5 "	20-38	++	++	+++	+++
	8 "	22-40	++	++ (1 cancer)	+++	+++
A	1 week	12-25	++	+	0	0
	2 "	14-40	++	++	+	+
	1 month	18-45	++	++	+	+
	2 "	15-36	++	++	++	++
	3 "	14-21	++	++	++	++
	5 "	15-28	++	++	++	++
	8 "	20-40	++	++ (1 cancer)	++	+++

* (+) minimal ductal hypertrophy and hyperplasia; + ductal hypertrophy and hyperplasia with secretion; ++ marked ductal hyperplasia with secretions.

† + alveolar budding; ++ multicentric adenomatous nodules.

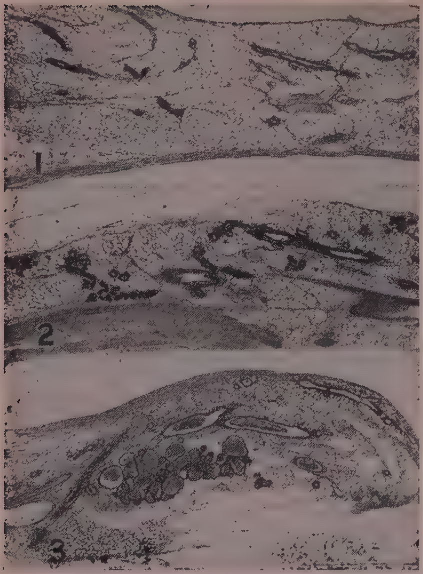
‡ + 7-12 layers of epithelium with keratinization; ++ 13 and more layers of epithelium with advanced keratinization; +++ downgrowth of epithelium into cervical stroma.

§ + glands enlarged; ++ glands cystic and hyperplastic with epidermization of cervical glands; +++ adenomatous glands.

strain Dba treated for one or 2 weeks, the Growth stimulation was of grade I; grade II changes were observed after treatment for one month (Fig. 4-6). Subsequently, epithelial growth became so active that after 5 or 8 months of injections, down growth into the stroma, closely resembling squamous carcinoma, occurred. In mice of strain A, the vaginal reaction was slowest: After one month, the growth stimulation was one of grade I, and only after 2 months, grade II stimulation had been reached.

Endometrium. In mice of strain C57, growth stimulation of grade II was seen after one injection of the estrogen, and after 2 months and later, polypoid growth was noted; however, no cancerous change was observed. In mice of strain Dba treated for one or 2 weeks, the growth stimulation was of grade I, and after one month, stimulation of grade II was observed; yet, growth was less marked than in the corresponding animals of strain C57. In mice of strain A, the endometrial reaction was even more delayed than in strain Dba, and did not reach grade II until after 2 months of treatment. At later stages of the experiment, there were no fundamental differences in the endometrial reaction, although mice of strain C57 reacted more vigorously than animals belonging to strains Dba or A.

Discussion. According to the present results and in agreement with the findings of Loeb (1,2), strain differences in the response to estrogen do not manifest themselves as a general susceptibility or non-susceptibility of the animals; instead, there are distinct differences in the sensitivity of the individual tissues, such as mammary gland, vagina and endometrium respectively. At the present time the nature of the factor or factors that determine the tissue susceptibility to estro-



A. Sections through the mammary glands of mice ovariectomized at the age of 3 weeks and subsequently injected with 0.03 mg of alpha estradiol benzoate once weekly for one month. ×20.

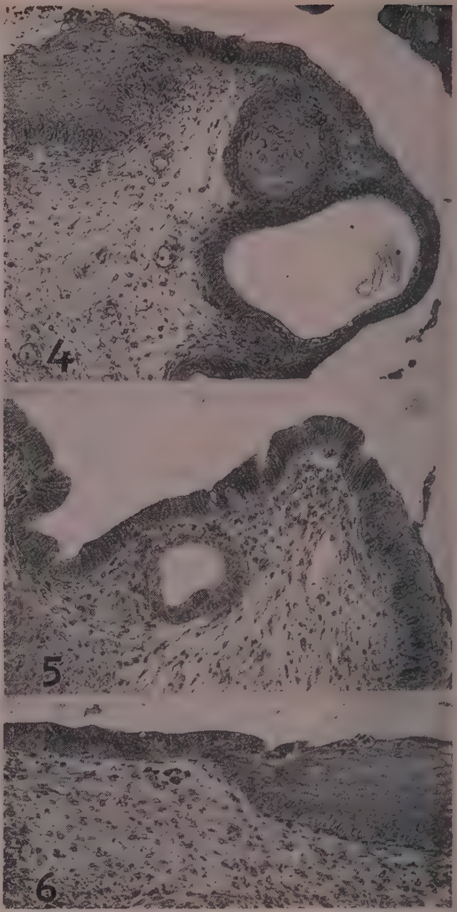
FIG. 1. Mouse of strain C57 black: Ductal growth minimal; alveolar growth 0.

FIG. 2. Mouse of strain Dba: Ductal growth grade II; alveolar growth grade I.

FIG. 3. Mouse of strain A: Ductal and alveolar growth grade II.

gen is unknown. The role of the milk agent in the mammary and vaginal response to estrogen has been repeatedly investigated, but the results are not unequivocal(5,9-11). Experiments are in progress in this laboratory which should help to determine the role of the milk agent in mammary growth preceding the development of cancer.

Summary. In ovariectomized mice of strains with different mammary cancer incidence (C57, Dba, A), injected with equal amounts of alpha estradiol benzoate, the response of mammary gland, vagina and endometrium respectively varied in degree: Mice of strain C57 showed less mammary stimulation than mice of strains Dba or A. The vaginal epithelium was most sensitive in mice of strain C57, slower in responding in mice of strain Dba, and least responsive in mice of strain A. The endometrium reacted most vigorously in mice of strain C57, and less actively in strains Dba and A. Differences in the mammary cancer incidence of the strains examined are considered to be due to differences in tissue susceptibility rather than to genetic differences in the output of estrogen.



B. Sections through the endocervix of mice ovariectomized at the age of 3 weeks and subsequently injected with 0.03 mg of alpha estradiol benzoate once weekly for one month. ×110.

FIG. 4. Mouse of strain C57 black: growth grade II with advanced epidermization.

FIG. 5. Mouse of strain Dba: growth grade II.

FIG. 6. Mouse of strain A: growth grade I.

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Effects of Low Concentration of Cyanide on Growth and Respiration in *Pelomyxa carolinensis*-Wilson. (18424)

D. M. Pace and B. W. McCashland.

From the Department of Physiology, University of Nebraska, Lincoln.

The inhibitory effect of cyanide on respiration in animal cells in general is now an accepted fact. Chiefly because of technical difficulties, the rather irregular results obtained in early investigations with protozoan cells gave the impression that ciliates were, perhaps, an exception in that they did not appear to be cyanide sensitive. One of the earliest reports on the effects of cyanide on protozoan species was that of Lund(1). He maintained that KCN did not affect respiration in *Paramecium caudatum*. These results appeared to be confirmed by those of Shoup and Boykin(2). Peters(3) did not find inhibition of respiration in *Colpidium colpoda* in solutions containing KCN, while Pitts(4) actually observed a 25% increase in oxygen uptake in *Colpidium campylum*. Lwoff(5) reported increases of as much as 30 percent above normal oxygen consumption by *Glaucoma piriformis* in M/1000 KCN in Ringer's solution. However, more recently, Boell working with *Paramecium calkinsi*(6), Pace with *P. caudatum* and *P. aurelia*(7), and Clark with *P. caudatum*(8) found that, when proper precautions are taken, cyanide inhibits respiration in these forms. In the latter investigations, balanced KOH-KCN absorption mixtures [Krebs(9)] were used in the flasks of the Warburg respirometer. Under these conditions, it was found that solutions of 10^{-5}M KCN concentration, or greater, inhibited respiration. The results indicate that much of the work reported previously was at fault in that these precautions were not taken and that cyanide was actually lost from the test solution by vaporization and absorption in the alkali. Pace and Belda(10) and Pace and Kimura(11) have shown that *Pelomyxa carolininsis* is also sensitive to cyanide. The sensitivity appears to be of the same order as that for the ciliate species. In solutions containing 10^{-5}M KCN or more, there is approximately a 65% inhibition in respiration.

The increase in respiration as found by Pitts and Lwoff(4) for certain protozoan species has not, as yet, been explained. Since they did not use KOH-KCN balanced absorption solutions it is evident that there must have been a continuous loss of cyanide from the solution containing the organisms. The final concentration of cyanide in solution may have been comparatively very low. The following investigation was carried out to ascertain the effects that low concentrations of cyanide might have on growth and respiration.

Materials and methods. The large multinuclear amoeba, *Pelomyxa carolinensis* Wilson, was used in this study. It was cultured in the special solution given in Table I.

Stock cultures of the organism were made up by adding approximately 150 ± 50 pelomyxae to 400 ml of the culture solution in stacking dishes of 20 cm diameter. Excellent growth occurred in 14 to 20 days if the

TABLE I.
Culture Solution for the Growth and Maintenance of *Pelomyxa carolinensis* Wilson.

K_2HPO_4	67 mg
NaH_2PO_4	39 ”
$CaCl_2$	100 ”
$MgCl_2$	22 ”
Distilled water to	1000 ml

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TABLE II. Effect of Low Concentrations of KCN on Growth of *Pelomyxa carolinensis* Wilson.

Molar conc. KCN	No. of cultures	Age of cells	Avg No. cells/culture after 7 days	% inhibition	% increase
0	3	Not recorded	166*		
10^{-5}	3	’’ ’’	90*	54	
0	23	Young	187		
10^{-7}	23	’’	181	3.7	
0	20	Old	156		
10^{-7}	20	’’	145	8.4	
0	18	Young	96		
10^{-9}	20	’’	131		49.3
0	20	Old	125		
10^{-9}	20	’’	142		17.0
0	19	Young	205		
10^{-11}	20	’’	218		6.3
0	20	Old	129		
10^{-11}	20	’’	128		0.9

* Cultures counted at the end of 6 days.

organisms in the cultures were fed every 4 days. The food organism, *Paramecium caudatum,* was grown in glass jars containing 500 ml of culture solution to which 0.2 g of proteose peptone (Difco) and 0.1 g of glucose had been added. After four to seven days, when the cultures were flourishing, 10 ml of the liquid containing the organisms were withdrawn from the edges at the top of the solution, filtered through loose cotton and by means of a hand centrifuge washed by centrifugation at the rate of 8 revolutions per second for 30 seconds. Paramecia contained in the equivalent of 10 ml were added to a pelomyxa stock culture at each feeding. These paramecia cultures could be used for feeding purposes for 3 to 5 weeks.

Results. Effect of low concentrations of cyanide on growth in pelomyxa. Growth tests were conducted as follows: 25 pelomyxae were added to 100 ml of the culture solution containing the equivalent of 10 ml of paramecium culture. For all tests, adequate portions of a concentration of washed paramecia which had been placed in a smaller amount of solution were added so that the number of paramecia was constant for all cultures. In those tests in which the highest concentrations of cyanide (10^{-5}M) were used no effort was made to prevent loss of cyanide by diffusion. However, for all other concentrations culture dishes were sealed to prevent excessive loss of cyanide as HCN. Results in lower concentrations of KCN showed that no appreciable difference in growth resulted when culture dishes were sealed with vaseline or placed in sealed desiccators above a cyanide solution 10 to 100 times the concentration of that used in the test solutions. Therefore, the results of all tests were tabulated together.

The cultures were placed in a dark chamber in which the temperature was maintained at 26 ± 2°C and did not change more than 1°C over a 24 hour period. The effect upon "young cells" was ascertained by using pelomyxae from 2- to 4-day stock cultures and for "old cells" stock cultures 10 to 12 days old were used. The number of pelomyxae in test cultures at the end of the growth period was ascertained by counting with a low power magnifying lens. The results are presented in Table II.

There is a definite inhibition of growth in *Pelomyxa* in solutions containing 10^{-5}M KCN as compared to the controls. In solutions containing 10^{-9}M KCN, there is a significant increase in growth when both "young" and "old" pelomyxae were used, although in 10^{-11}M KCN, there is no significant difference.

Aside from cyanide itself, potassium was the only other variable in the above tests. Because of the possibility that this might have had an effect on growth the following tests were carried out. In these tests potassium chloride was added to the solution in place of potassium cyanide and in the same concentrations. The results are given in Table III.

TABLE III. Effect of Low Concentrations of KCl on Growth of *Pelomyxa carolinensis* Wilson.

Molar conc. KCL	No. of cultures	Age of cells	Avg No. cells/culture after 7 days	% inhibition	% increase
0	8	Young	122		
10^{-7}	4	”	124		2.1
0	4	Old	85		
10^{-7}	5	”	84	1.7	
0	8	Young	122		
10^{-9}	4	”	125		3.1
0	4	Old	85		
10^{-9}	4	”	89		6.7
0	5	Young	204		
10^{-11}	5	”	202	0.9	
0	4	Old	85		
10^{-11}	5	”	85		

The results are remarkably similar in the controls to those of the test cultures. The slight differences that do exist are not significant. It is evident that very low concentration of potassium has very little, if any, effect on growth in pelomyxa.

Effect of low concentrations of cyanide on respiration in pelomyxa. Pace and Belda(10) found that potassium cyanide inhibits respiration in *Pelomyxa;* the lowest concentration tested by them was 10^{-5}M. Because of the results obtained on growth with much lower concentration of cyanide as reported above, it seemed expedient that observations be made upon the effect of these low concentrations on respiration. The Barcroft-Warburg micro-respirometer was used for this study. Pelomyxae were counted and washed in fresh solution containing the desired concentration of KCN (Table I) the day before each test. They remained in this solution overnight. At the time of the test, the organisms were washed again and placed in the Warburg flask in 5.0 ml of the solution. Into the side-arm of each flask 0.3 ml of M HCl was introduced. The center well contained 0.2 ml of the proper KOH-KCN mixture.* Observations were made over a period of from 4 to 6 hours for each test. The results of these experiments are presented in Table IV.

Respiration is inhibited by about 50% in 10^{-7}M KCN and by 15% in 10^{-9}M. A definite increase in respiration is shown by *Pelomyxa* in the lowest concentration (10^{-11}M KCN) used in these studies. The increase in rate of respiration seems to be greater for the "old" pelomyxae than for the "young" which is just the reverse of that obtained in the growth studies. The significance of this difference is as yet not known.

Discussion. Pace and Belda(10) and Pace and Kimura(11) used cyanide in concentrations that inhibited respiration in *Pelomyxa,* and from their results, it appears that up to 70% of its oxygen consumption is dependent upon a cytochrome-cytochrome oxidase system. Results presented in this paper indicate that growth processes are also inhibited in 10^{-5}M KCN. This might be anticipated since general metabolic activity including growth would be affected by loss of the CCO system.

The increased respiration in low cyanide concentration, (10^{-11}M KCN), offers some evidence to indicate that increased respiration in ciliates as observed by Pitts(4) and Lwoff (5) might possibly have been due to the fact that proper precautions were not taken to prevent excessive cyanide loss. Thus, as more cyanide was lost from their test solutions, the concentration approached the level which caused accelerated respiration, or at least did not inhibit it. That this effect is due to cyanide, and not potassium ion concentration, is demonstrated by substituting KCl for KCN.

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*The KCN and KOH concentrations of the absorption solution varied with the KCN concentration of the experimental culture fluid as follows:

Molar conc. of KCN in culture sol.	Absorption solution in inset
10^{-7}	10 ml M KOH; .05 ml M KCN
10^{-9}	10 ml M KOH; .01 ml M KCN
10^{-10} or 10^{-11}	10 ml M KOH; .01 ml M/10 KCN

TABLE IV. Effect of Low Concentration of KCN on Respiration in *Pelomyxa carolinensis* Wilson.

Molar conc. KCN	No. of tests	Duration of tests	Avg O_2 consumption, in mm^3/million/hr	% inhibition	% acceleration
0	3	4 min.	12060		
10^{-7}	3		5930	50.9	
0	13	4 hr	7430		
10^{-9}	11		6290	15.3	
0	3	6 hr	4030		
10^{-10}	3		4290		6.5
0*	9	4 hr	13670		
10^{-11}*	7		18140		32.7
0†	10	4 hr	9450		
10^{-11}†	12		14115		49.3

* Young cells, 2-3 days.

† Old cells, 7 days.

There is a direct relationship between respiratory processes (furnishing energy) and processes by which new protoplasm is synthesized. One would therefore expect that inhibitions or accelerations of growth or respiratory processes would occur at similar concentrations of cyanide. The fact that this did not hold true in these investigations may be due to some loss of cyanide from the solution in the dishes used for studying growth. Therefore, the concentration of cyanide put into the solution that supported maximum growth (10^{-9}M KCN) is probably not the concentration that exists after the dishes have been sealed for a few hours. It possibly approaches the concentration that was found optimum for oxygen consumption (10^{-11}M).

Further investigation is necessary to attempt to ascertain the reason for acceleration of growth and respiration in low concentrations of cyanide, although several hypotheses may be stated from the data obtained. For example, there is some evidence that cyanide is detoxified and at the same time has a reducing action on glutathione [Voegtlin, Johnson, and Dyer(12)]. This reduction may enhance the action of glutathione as an agent associated with the respiratory process. Such an action would not be evident in higher concentrations of cyanide because the increased action of glutathione would be nullified by the great decrease in the action of the CCO mechanism. The presence of glutathione in *Pelomyxa* was suggested by the work of Pace and Belda(10) and also by positive nitro-prusside tests (unpublished results). It has been suggested that about 30% of the respiration in *Pelomyxa* is associated with glutathione.

It is also possible that there may be regulatory mechanisms within cells which tend to retard action in other systems and which are even more sensitive to cyanide than the CCO system. These mechanisms would be inactivated first and if the concentration of cyanide is low enough there would be no action on cytochrome oxidase, resulting in an actual increase in metabolism. It is hoped that our knowledge on the possible mechanisms involved will be enlightened by future work.

Summary. 1. Concentrations of KCN from 10^{-7}M and higher cause inhibition of growth in *Pelomyxa carolinensis*. 2. In solutions containing 10^{-9}M KCN there is on the average approximately a 50% increase in growth in "young" *Pelomyxa* and a 17% increase in "old" *Pelomyxa*. 3. These effects are not caused by increase or decrease in potassium concentration. Potassium, alone, has very little effect, if any, in very low concentration. 4. Concentrations of KCN from 10^{-9}M and higher cause inhibition of respiration in *Pelomyxa*. 5. In solutions containing 10^{-11}M KCN, there is an approximate 32 and 50% increase in rate of respiration in "young" and "old" pelomyxae respectively, as compared to controls without cyanide.

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Localization of Acetylcholinesterase Activity in the Protozoan, *Tetrahymena geleii* S. (18425)

GERALD R. SEAMAN.
From the Carter Physiology Laboratory, University of Texas Medical Branch, Galveston, Tex.

The presence of a specific acetylcholinesterase (AChE) in *Tetrahymena* has been demonstrated(1). It was shown that specific inhibition of the activity of the enzyme results in decreased motility of the organism. The suggestion was offered that the fibrillar structure in the pellicle of the ciliate serves as a primitive conducting mechanism analogous to nerve fibers, and as such, is dependent upon AChE activity for function. If this be true, all of the AChE activity of the cell should be localized in the pellicle, in the same fashion that the total activity of this enzyme is localized in the sheath of nerve fibers(2).

Methods. Cells of *Tetrahymena geleii* S were grown in pure culture, and were harvested and washed as described previously(1). The resulting cell concentrate was homogenized in 0.25 M sucrose with a Potter-Elvehjem grinder. The homogenate was centrifuged at 600 X *g* for 10 minutes. The supernatant, fraction S_1, was removed and the sediment washed twice by rehomogenizing, each time with 0.25 M sucrose, and centrifuging at 600 X *g* for 10 minutes. The supernatants were removed and added to fraction S_1. At this point, only nuclei, pellicular fragments and a few unbroken cells are contained in the sediment. Phase contrast examination (bright contrast) of this fraction reveals none of the cytoplasmic inclusions described for *Tetrahymena*(3). A force of 600 X *g* is obviously not sufficiently great to separate the small cytoplasmic particles; they are retained in fraction S_1. The residue was then suspended in 0.25 M citric acid and allowed to stand for 15 minutes. The mixture was then centrifuged at 600 X *g* for 10 minutes. The nuclei remain in the supernatant while pellicular material is sedimented. The supernatant was removed and was labeled fraction N. The sediment was resuspended by homogenizing with a pinch of quartz in a buffer consisting of 0.05 M Na_2HPO_4, 0.1 M NaCl, 0.02 M $MgCl_2$ (pH 7.0). This procedure brings the AChE into solution(4). The mixture was then centrifuged at 1600 X *g* for 20 minutes to remove all particulate matter. The supernatant, fraction S_2, contained no microscopically visible particles. The entire fractionation procedure was carried out at 4°C. AChE activity of each fraction was estimated by measuring the rate of removal of added acetylcholine (ACh). ACh was determined by the colorimetric method of Hestrin(5). Aliquots of the original homogenate were dried to constant weight at 80°C and the enzymatic activity was expressed as Q_{AChE}, μg ACh split/hour/mg dry weight of original homogenate.

Results and discussion. Table I shows that all the AChE in *Tetrahymena* is contained in fraction S_2 (the pellicular fraction). No significant esterase activity was obtained with the first supernatant, S_1, or the residue, fraction R.

TABLE I. Distribution of AChE Activity in Centrifugal Fractions of *T. geleii* S. Figures in parentheses indicate range obtained in 7 determinations. Figures in *italics* mean of values obtained.

Q_{AChE}	Fraction
(.067-.082) *.078*	Entire homogenate
(.002-.006) *.003*	S_1
(.043-.076) *.072*	S_2
(.001-.003) *.003*	N
(.000-.003) *.002*	R

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These results thus lend support to the hypothesis(1) that conduction along the fibrillar structure which connects the base of individual cilium in the pellicle of the organism is similar to conduction along nerve fibers, and is dependent upon AChE activity for function(6).

Attempts were made to localize, by histochemical methods, the AChE activity of *Tetrahymena*. It was thought that such procedures would visualize a pattern on the pellicle which would be identical with the pattern of the fibrillar system as revealed by the silver technic(7). Cells were therefore treated according to the procedures for histochemical localization of AChE as described by Gomori(8) and as by Koelle(9). The stain however, in all cases, diffused to a great extent and became located in cytoplasmic granules, which actually contained no AChE activity (Table I).

Summary. AChE activity in the ciliate *Tetrahymena geleii* S is localized in the fibrillar system of the pellicle. Upon differential centrifugation, a fraction was obtained which contained only enzymatic activity from the pellicle. This fraction contained the entire amount of AChE activity of the cell.

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Implantation of Ureters into Completely Isolated Loops of Small Intestine.[*] (18426)

Walter L. Mersheimer, Albert J. Kolarsick, and Henry Kammandel.
(Introduced by Israel S. Kleiner.)

From the Department of Surgery, New York Medical College and Flower and Fifth Avenue Hospitals.

The problem of constructing an artificial urinary bladder has become increasingly more urgent because of the performance of large numbers of radical operations, particularly pelvic viscerectomy.

Reports have appeared concerning the use of partially or completely isolated loops of small intestine to serve as a urinary reservoir (1-4). Our method of performing uretero-ileal-cutaneous implantation has not previously been reported and our investigation would indicate that it is superior to the older technics.

The procedure we have successfully performed is as follows: With the dog under intravenous sodium pentothal anesthesia, a left rectus muscle splitting incision is used to enter the abdomen. A loop of terminal ileum approximately 10 cm in length is isolated and the continuity of the small intestine is restored by end to end anastomosis. The proximal end of the isolated ileal loop is closed by the Parker-Kerr technic. The ureters are transected close to the bladder and the distal ends ligated. The ureters are implanted through the same seromuscular tunnel in the antimesenteric border of the isolated loop of the intestine. The distal open end of the

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[*] Aided by a Grant from the Research Fund of the New York Medical College.

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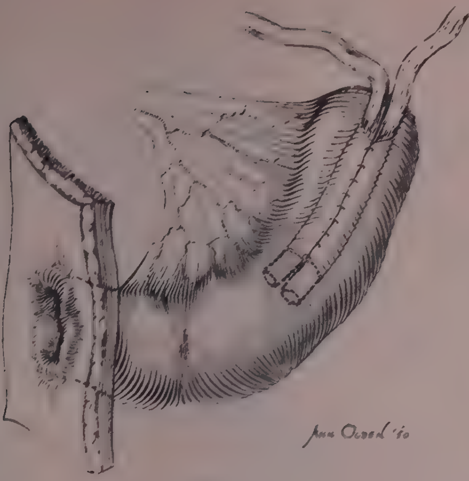

FIG. 1.

loop is brought through a stab wound in the abdominal wall as an ileostomy. The method is illustrated in the accompanying diagram.

In the small series of animal experiments there has been survival up to 18 months. Sacrifice of the 18-month dog and postmortem examination showed normal kidneys and pelvis without evidence of infection, and slightly dilated ureters. The isolated loop of ileum, the uretero-intestinal anastomoses and the ileostomy all appeared normal. Microscopic sections of the kidney did not show any significant lesions. Studies of the blood nonprotein nitrogen and urea nitrogen, urinalyses and intravenous pyelography prior to sacrifice of this dog were within normal limits.

A similar method was used to transplant the left ureter on two additional dogs. At the time of re-operation the left kidney and ureter compared favorably with the non-operated right kidney and ureter, both sides appearing within normal limits.

Conclusion. A new and successful method of uretero-ileal-cutaneous implantation is described. The method offers advantages: (1) no contact of ureteral orifices with the fecal stream, which should reduce the incidence of infection; (2) it avoids the undesirable features of a wet colostomy, and should be applicable to the patient undergoing pelvic viscerectomy.

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Hemagglutination of Tuberculin Sensitized Sheep Cells in Hansen's Disease (Leprosy). (18427)

MAX LEVINE.

From the Bureau of Laboratories, Territorial Department of Health, Honolulu, Hawaii.

The hemagglutination test of Middlebrook and Dubos(1), as modified by Scott and Smith(2), provides a relatively readily available laboratory tool for studying the serological response of patients infected with tubercle bacilli. The following brief report indicates that this technic may be especially applicable for measuring a serological response in Hansen's disease.

Materials and methods. The technic employed for determination of hemagglutination titers was that described by Scott and Smith except that 0.25% sensitized sheep red cell suspensions were employed; the reacting volume was 1.0 ml compared with 0.8 ml in their report; and the initial test titer was 1:4. Some observations were also made on the effect of agitating the reacting mixture in a Kahn shaking machine for 15 minutes, then centrifuging and immediately reading, on the agglutination titer as compared with the technic of Middlebrook and Dubos and Scott and Smith which consists of incubation for 2 hours at 37°C followed by standing overnight before reading. There was an excellent correlation between the two methods of determining the

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TABLE I. Agglutination of Tuberculin Sensitized Sheep Red Cells by Sera from Tuberculosis and Hansen's Disease Patients and Blood Donors.

			Hansen's disease patients	
Source of specimens	Leahi* T.B. hospital (96 patients)	Blood bank donors (40 individuals)	80 active cases (bact. pos.)	25 released cases (bact. neg.)
Agglutination titer	% of specimens			
Neg. 1:4	11.5	75.0	1.3	24
1:4	11.5	15.0	8.7	32
1:8	11.5	7.5	13.7	16
1:16	28.1	—	17.5	16
1:32	14.6	2.5	11.3	4
1:64	17.7	—	17.5	—
1:128	5.2	—	11.3	8
1:256	—	—	8.7	
1:512	—	—	6.2	
1:1024	—	—	2.5	
1:2048	—	—	1.3	

* Clinical status of activity of disease in these patients undetermined at this time.

hemagglutination titers, and in our hands the centrifuge method was easier to read, but the titers reported herein are those obtained by the previously described technic.

Specimens from 96 tuberculous patients were obtained from the Leahi Tuberculosis Hospital in Honolulu and sera from 105 Hansen's disease patients were obtained at the Hansen's Disease Hospital (Hale Mohalu) in Pearl City, Oahu, Hawaii. The latter included 80 individuals in the active phases of the disease, all of which are bacteriologically positive, and 25 individuals who had been released as arrested cases, and all of which were bacteriologically negative. (Only 3 of the Hansen's disease patients were also T.B. cases—2 were bacteriologically positive for Hansen's and negative for T.B. organisms and one negative for both.) A control group of 40 specimens was obtained from blood donors at the Blood Bank of Hawaii.

Results. As may be seen from the accompanying table and graph, the low titers of the control group and the frequently very high titers in Hansen's disease patients, who are bacteriologically positive, stand out in marked contrast. The lower intermediate titers of bacteriologically negative Hansen's disease patients, in whom the disease has been arrested, are also very striking. Thus, titers of 1:4 or less were obtained in 90% of blood donors as compared with only 10% in Hansen's disease patients who were bacteriologically positive, but in 56% of released, bacteriologically negative, patients.

Only one (2.5%) of the 40 control specimens gave a titer of over 1:8, and only 2 (8.0%) of the arrested, bacteriologically negative, Hansen's disease patients had titers over 1:32. In contrast to this, 5 (5.2%) of the tuberculosis sanatorium and 9 (11.3%) of the active, bacteriologically positive, Hansen's disease patients showed agglutination titers of 128 and in addition 15 (18.7%) of the Hansen's disease cases had titers of 256 to 2048.

To cogitate on the possibility that the acid-fast bacteria associated with tuberculosis and Hansen's disease might have common antigenic components is, of course, not surprising; but to verify such an hypothesis, in the absence of pure cultures of the latter, was not feasible by the generally employed procedures. The hemagglutination technic of Middlebrook and Dubos provides an indirect approach toward an answer to this hypothesis. The marked serological response to antigens present in tuberculin* was as unanticipated as it is striking. This tool (agglutination of tuberculin sensitized red cells) opens up new vistas for study of that disease of antiquity

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* Appreciation is expressed to Dr. H. D. Piersma of the Lederle Laboratories for supplying us with the 4X tuberculin employed in these studies.

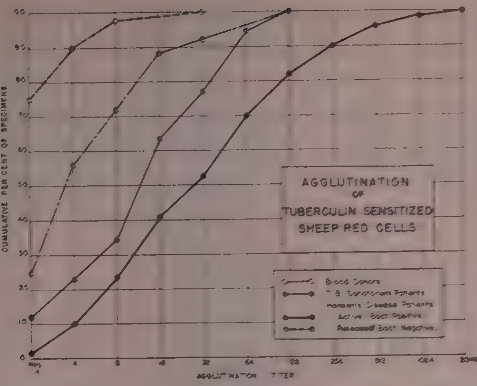

FIG. 1.

whose etiological agent still defies cultivation.

It seems evident that *Mycobacterium tuberculosis* and *Mycobacterium leprae* possess common antigens which stimulate production of common agglutinins in human sera. This does not necessarily indicate a corresponding immunological response. However, the possibility that such a relationship may exist (it does, for example, with respect to various strains of *Salmonella*, as indicated by Longfellow and Luipold(3) and Levine *et al.*(4) raises an intriguing conjecture as to the efficacy of B.C.G. vaccination of children of Hansen's disease patients as a protective measure.

On the basis of observations that B.C.G. vaccination induces a positive lepromin test, Chaussinand(5) suggests that it would be of interest to determine whether a preventive action against leprosy is also produced. The data presented, herein, on the serological response of Hansen's disease patients to antigens in tuberculin, serve to augment the view that observations on the heterologous immunological effects of B.C.G. vaccination, with particular reference to Hansen's disease, are worthy of careful consideration.

Summary. The hemagglutination titers of active, bacteriologically positive, cases of Hansen's disease were significantly higher than those of arrested, bacteriologically negative, cases of this disease, and frequently much higher than those encountered in the active cases of tuberculosis examined. The immunological response against antigenic components of the tubercle bacillus elicited by infection with Hansen's disease appears to be very marked and distinct. The inability to grow *Mycobacterium leprae* has naturally hampered serological studies of Hansen's disease. The hemagglutination technic of Middlebrook and Dubos provides a laboratory tool which may be very useful in future studies of this entity.

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3. Longfellow, D., and Luipold, G. F., *Am. J. Hyg.*, 1943, v38, 139.

4. Levine, M., Enright, J. R., Ching, G., and Tanimoto, R., *Hawaii Med. J.*, 1950, v9, 149.

5. Chaussinand, R., *Int. J. Leprosy*, 1950, v18, 79.

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Effect of APF in Supplying Multiple Factors for the Pig.* (18428)

H. M. EDWARDS, T. J. CUNHA, G. B. MEADOWS, C. B. SHAWVER, AND A. M. PEARSON.
(Introduced by Paul B. Pearson.)

From the Department of Animal Husbandry and Nutrition, Florida Agricultural Experiment Station, Gainesville.

Using a corn-peanut meal ration Cunha *et al.*(1) showed that APF (Lederle) and B_{12} were different. This difference was later found by Jukes *et al.*(2) to be at least partially caused by the presence of aureomycin in the APF (Lederle). The purpose of the

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* This study was supported in part by a grant from Swift & Co., Chicago.

1. Cunha, T. J., Burnside, J. E., Buschman, D. M., Glasscock, R. S., Pearson, A. M., and Shealy, A. L., *Arch. Biochem.*, 1949, v23, 324.

2. Jukes, T. H., Stokstad, E. L. R., Taylor, R. R., Cunha, T. J., Edwards, H. M., and Meadows, G. B., *Arch. Biochem.*, 1950, v26, 324.

TABLE I. Data on the Effects of Vitamin B_{12} and Aureomycin Mash Concentrate, With or Without Alkali Treatment, on Growth of Pigs Fed a Corn Peanut Meal Diet.

Group No.	Supplement	Exp. 1		Exp. 2	
		Avg starting wt, lb.	Avg daily gain, lb.	Avg starting wt, lb.	Avg daily gain, lb.
1	0	49.5	0.79	30.1	0.98
2	Vit. B_{12}*	47.7	1.18	29.9	1.27
3	Alkali-treated aureomycin mash concentrate†	48.1	1.40	29.1	1.41
4	Vit. B_{12} + alkali-treated aureomycin mash concentrate†	47.4	1.23	29.1	1.43
5	Vit. B_{12} + alkali-treated aureomycin mash concentrate, high level‡			29.5	1.48
6	Aureomycin mash concentrate§	[illegible]	[illegible]	[illegible]	[illegible]

* B_{12} concentrate which contained approximately equal parts of vit. B_{12} and B_{12b}, obtained from Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N. Y. The B_{12} was mixed in the feed at a level of 44 µg per kilo of diet.

† The liquid alkali-treated concentrate was fed at a level of 22 ml per kilo of diet in Exp. 1, derived from 20 ml of untreated concentrate, and 33 ml in Exp. 2, derived from 33 ml of untreated concentrate. The treatment with alkali consisted of steaming for 30 min. in 1.0 N NaOH. The alkali-treated concentrate contained 30% to 35% NaCl which was produced by neutralization of the sodium hydroxide used in the treatment, so that NaCl was added to the other diets to bring the level up to the total NaCl in lot 4.

‡ 66 ml per kilo of diet.

§ Prepared by concentrating aureomycin mash and fed at a level of 30 ml per kilo of diet in Exp. 1 and 22 ml in Exp. 2.

experiments reported herein was to study the multiple nature of the growth stimulating property of the Lederle APF.

Materials and methods. The control ration used in these experiments consisted of corn, peanut meal, minerals and vitamins as previously described by Cunha *et al.*(1) except that a level of 0.53% of a salt-trace mineral mixture was used instead of 0.5% salt. This mixture consisted of iodized salt 50 lb., manganese sulfate 800 g, ferrous sulfate 398 g, copper sulfate 125 g, and cobalt carbonate 16 g. Four Duroc pigs were used in each lot in both experiments. Exp. 1 was conducted for 59 days and Exp. 2 for 52 days. The pigs were fed on concrete floors which were washed once daily. Three of the 4 pigs used in each lot in Exp. 1 were 14 weeks old and had been depleted on the basal ration for 6 weeks before being started on experiment. The fourth pig in each lot in Exp. 1 was 11 weeks old and had been depleted for 3 weeks on the basal ration before being started on experiment. The pigs used in Exp. 2 were 67 days old and had been depleted for 11 days on the basal ration before being started on experiment.

Results and comments. The data obtained in Exp. 1 and 2 (Table I) show that vit. B_{12} was of benefit for pigs fed the corn-peanut meal basal ration, when the pigs had been previously depleted. The pigs in Exp. 1 which had been depleted longer gave the greater response to vit. B_{12}. Previous data by Cunha *et al.*(1,3) and Burnside *et al.*(4) have shown that weanling pigs (8 weeks old and not depleted) have responded very little, if at all, to B_{12} on a corn-peanut meal ration.

Treatment of aureomycin mash with NaOH, as described, was found(5,6) to destroy vit. B_{12} completely and to destroy 97% of the inhibitory effect of aureomycin against *Staphylococcus aureus*, but the material retained antibiotic activity against various other microorganisms. The data obtained in the present investigation indicate that the alkali-treated material contained a factor or

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3. Cunha, T. J., Hopper, H. M., Burnside, J. E., Pearson, A. M., Glasscock, R. S., and Shealy, A. L., *Arch. Biochem.*, 1949, v23, 510.

4. Burnside, J. E., Cunha, T. J., Edwards, H. M., Meadows, G. B., LaMar, G. A., Pearson, A. M., and Glasscock, R. S., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 173.

5. Jukes, T. H. Personal communication, 1950.

6. Stokstad, E. L. R., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 523.

factors which increased the rate of growth of pigs. The same increase was observed with or without the addition of vit. B_{12} which may indicate that the factor or factors in the alkali-treated material have a sparing effect upon vit. B_{12}. Whether or not crystalline aureomycin would have this sparing effect was not determined.

Summary. The data obtained show that B_{12} is beneficial for pigs which were previously depleted on a corn-peanut meal ration. They also show that alkali-treated APF contains a factor or factors which promote growth of pigs and which may have a sparing effect upon B_{12} needs of the pig.

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Biochemical Studies on the Iguana. (18429)

Thomas Hernandez and Roland A. Coulson.

From the Department of Biochemistry, Louisiana State University School of Medicine, New Orleans, La.

Although the iguana is one of the most common reptiles of tropical America, very little is known of the biochemical nature of these animals. Two species of Iguanidae were obtained from a biological supply house; 7 specimens were "black iguanas," *Ctenosaura acanthura,* from 80-93.5 cm and weighing 830-990 g, and 9 were "common iguanas," *Iguana iguana rhinolopha,* ranging from 111.5-148 cm and weighing 900-1910 g. The common iguana is easy to handle and harmless whereas the black iguana is more active and has to be handled with some caution because of attempts to bite, or to scratch with its sharp hind claws. Both species subsisted on a diet of cabbage.

The analyses reported here were done in August and September. Blood was obtained by cardiac puncture using a No. 19 gauge needle. Best results were obtained by holding the iguana on his back and inserting the needle in an intercostal space on either side of the thorax 1-2 cm caudal to the front limbs. Although some animals received as many as 10 cardiac punctures only 2 died as a result of this procedure. Urine was obtained by inserting a catheter along ventral wall of cloaca. The analytical methods employed were the same as those reported for the alligator studies(1,2), except for plasma magnesium content which was done by the method of Denis(3).

Results. Table I presents a summary of the general blood studies. The oval nucleated erythrocytes are of large size, the mean corpuscular volume (M.C.V.) for the black iguana being 338 μ^3 and for the common iguana 298 μ^3. In comparison, the alligator corpuscles are larger(1), having an M.C.V. of 472 μ^3. Table II reveals a number of differences between the two species of lizards in fasting blood and plasma CO_2, chloride, and pH values. Although the CO_2 and chloride contents are different for the two the sums of the anions are about the same. The lower plasma CO_2 of the black iguana is in keeping with the lower blood pH. The differences between the two in the plasma cations are probably not significant. Both species have plasma phosphorus concentrations which are considerably higher than those of the alligator.

The results of the urine analyses of the common iguana are summarized in Table III. In contrast to the alligator the pH of the urine is always more acid than the blood of the same animal and only 6 of the 36 determinations were above pH 7. There was only an occasional trace of chloride present. The values obtained for uric acid, the chief nitrog-

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2. Coulson, R. A., Hernandez, T., and Dessauer, H. C., Proc. Soc. Exp. Biol. and Med., 1950, v74, 866.
3. Denis, W., *J. Biol. Chem.,* 1922, v52, 411.

TABLE I. Blood Studies.

	No. of animals		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	Avg	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
		Range	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Common iguana	9	Avg	1.66	29	9.5	7.1	295	68	22
		Range	0.72-1.28	25-36	5.1-16.1	3.8-12.0	257-366	[illegible]	[illegible]

TABLE II. Composition of Blood and Plasma.

	No. of animals		[illegible] mg %	pH	[illegible] mM/L	[illegible]	[illegible]	[illegible]	[illegible]
			A. Composition of whole blood.						
[illegible]	[illegible]	Avg	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
		Range	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Common iguana	[illegible]	Avg	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
		Range	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
			B. Composition of plasma.						
			Cl, mM/L	CO_2, mM/L	Na, mM/L	K, mM/L	Ca, mg %	P, mg %	Mg, mg %	Pr., %
[illegible]	[illegible]	Avg	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
		Range	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Common iguana	[illegible]	Avg	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
		Range	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

TABLE III. Composition of Urine of the Common Iguana.

	No. of determinations	Avg	Range
pH	36	6.14	5.69-7.45
CO_2, mM/L	9	12.9	4.9-31.7
Cl, "	9	tr.	0 -tr.
Na, "	9	26.6	14.5-46.6
K, "	9	45.8	15.3-55.2
NH_3, mg %	22	12.1	1.0-32.8
Urea, "	22	10.9	0-87.2
Uric acid, mg %	15	132.5	40.6-247.7
Creatinine, "	29	3	0.9-6

enous end product, vary widely and depend on the amount of solid uric acid and urates removed from the bladder by catheterization. Two composite specimens of dried excreta were analyzed for uric acid, sodium, potassium, and calcium. Of the 2 specimens analyzed for uric acid one gave a value of 10.3% and the other 96.4% of dry weight of the excreta. The concentration of the constituents of the excreta is dependent apparently on the amount of undigested organic matter present and is therefore highly variable. The calcium was 8% and 21%, sodium 0.4% and 4.5%, and potassium 8% and 23% of the ashed weight of the 2 specimens.

Two iguanas were dried, then ashed at 500°C in an electric furnace. The results of the ash analyses are recorded in Table IV. The volume of the extracellular fluid may be estimated crudely by assuming all of the chloride found in the ash to have originated in the extracellular fluid. On this assumption, the extracellular fluid of iguana No. 4 was 24.7% and of No. 8, 26.3% of body weight. Calculation of the extracellular fluid volume on the basis of the sodium content of the ash gives values which are in good agreement with the above.

Since the alligator shows a pronounced alkaline tide(2), it was of interest to see whether this might also be true of the iguana. Two common iguanas were force fed with skinned rats and the blood pH and plasma chloride, CO_2, and sodium determined at 2 hr., 4½ hr., 11 hr., and 23 hr. after feeding. There were no differences in values between the experimentals and 2 controls used. Apparently the pH and electrolyte changes, if any, are too small to be measurable. Similarly, experiments with histamine on the alkaline tide met with no success. Four iguanas were injected I.M. with dosages up to 2

TABLE IV. Composition of Iguana Ash.

Iguana No.	Sex	Wt, g	Dry wt, g	H_2O, %	Ash, g	Cl, mg	Na, mg	K, mg	Ca, g	Mg, g
4 (common)	F	932	252.3	72.9	57.88	1144.	[illegible]	1349.4	18.2	[illegible]
8 (black)	M	867	253.4	70.8	50.23	1008.7	900.4	1340.2	15.75	.427

mg kilo, and blood pH and plasma chloride, CO_2, and sodium were determined 1, 2, 3, 4, 11, and 23 hours after injection. The values obtained were of the same order of magnitude as those of the controls.

Summary. Two species of large lizards, *Iguana iguana rhinolopha* (common iguana) and *Ctenosaura acanthura* (black iguana) were studied. The following analyses were performed on blood and/or plasma of both species: pH, CO_2, Cl, Na, K, Ca, Mg, protein, urea, uric acid, creatinine, glucose, hematocrit, oxygen capacity, and mean corpuscular volume. There was little difference in the average values for the two species with the exception of pH, CO_2, and chlorides. Although the total ionic concentration of the plasma was about the same for both, the Common Iguana appeared to be more alkaline than the Black Iguana. The urines and total ashes were analyzed for Na, K, Ca, Mg, and chloride. Uric acid, Na, K, and Ca analyses were done on dried excreta.

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Anatomic and Histologic Changes in Rabbits with Experimental Hypersensitivity Treated with Compound E and ACTH. (18430)

FREDERICK G. GERMUTH, JR., GLEB A. NEDZEL, BARBARA OTTINGER, AND JIRO OYAMA.
(With the technical assistance of Kenneth Grow.)
(Introduced by Karl Habel.)

From the Microbiological Institute and the Experimental Biology and Medicine Institute, National Institutes of Health, Public Health Service, Bethesda, Md.

Previous work(1) has indicated that Compound E and ACTH inhibit the production of experimental hypersensitivity of the Arthus type in rabbits receiving daily intracutaneous injections of crystalline egg albumin. This inhibition results from the hormonal suppression of antibody formation. The present report describes the anatomic and histologic changes produced in these animals by Compound E and ACTH with emphasis on the histologic appearance of the Arthus reaction and on the organs possibly related to antibody formation.

Methods and materials. Twenty-four male albino rabbits weighing approximately 2 kg each were injected intracutaneously every day for 18 days with 0.2 ml of crystalline egg albumin solution(2) containing 0.8 mg of egg albumin nitrogen. Simultaneously, 8 of the 24 animals were treated daily with a single intramuscular dose of 10 mg of Compound E and another 8 with 10 mg of ACTH per day administered in 4 intramuscular doses of 2.5 mg every 6 hours. Two to 3 ml of blood were drawn from the marginal ear vein at the beginning of the experiment and on the 4th, 7th, 9th, 11th, 14th and 16th days for the antibody studies previously reported (1). Total white blood counts and differential counts were performed on the 17th day. Eighteen days after the first injection of egg albumin, the surviving animals were bled from the heart and killed. The weights of the thymus, spleen, liver, adrenals and kidneys

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2. Kekwick, R. A., and Cannan, R. K., *Biochem. J.*, 1936, v30, 227.

were recorded; in addition, sections for microscopic study were taken from the thyroid, thymus, lungs, heart, liver, spleen, pancreas, mesenteric nodes, adrenals, kidneys, testes, leg muscle, femoral marrow and skin at the site of the final injection of egg albumin, and fixed in 10% formalin. All sections were stained with azure-eosin(3). The liver, kidneys, adrenals and hearts were also stained in frozen section with oil red O to demonstrate fat(3), and liver sections of the Compound E-treated and control animals, which had been previously fixed in absolute alcohol, were stained with Best's carmine stain for glycogen. Quantitative glycogen determinations were carried out on weighed portions of the dried alcohol-fixed liver by hydrolyzing the alcohol precipitated glycogen to glucose and measuring the latter according to the method of Hagedorn and Jensen(4). In addition to the 24 experimental animals 5 normal rabbits were autopsied for comparison with the sensitized, untreated controls.

Six of the 24 experimental rabbits died before the experiment was terminated. Four had been treated with Compound E, one with ACTH, and one was an untreated control. All of these animals were affected with severe diarrhea. Another ACTH-treated animal was killed because of diarrhea. Except for these 7 animals which became extremely dehydrated and emaciated prior to death, the rabbits maintained their weight or showed an increase in weight during the experimental period. It is interesting that the rabbits receiving Compound E became increasingly sluggish and irritable after the first several days of treatment. At the same time, their muscles appeared flabby and their abdomens were protuberant. In connection with the serological studies it was observed that the blood of these animals became considerably lipemic. These alterations were not seen with ACTH.

Results of pathological studies. A. Rabbits injected with egg albumin and receiving no treatment (control animals). After a preliminary period of sensitization, 6 of 8 control animals produced large, hemorrhagic and necrotic Arthus reactions. Sections of these lesions showed (a) marked edema, hemorrhage, and leucocytic infiltration of the derma, (b) necrosis and acute inflammation of the subcutaneous muscle, and (c) focal fibrinoid necrosis, acute inflammation, and thrombosis of the blood vessels, particularly of the small arteries lying between the derma and the subcutis. These vascular lesions were similar in appearance to those seen in periarteritis nodosa. Two of the 8 rabbits produced extensive erythema and edema in response to the final injection of the antigen. Microscopic

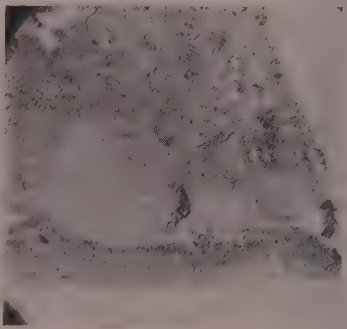

FIG. 1.

Section of derma and subcutis of a control rabbit at site of Arthus reaction showing hemorrhage, edema, leucocytic infiltration, and thrombosis of blood vessels. 10×.

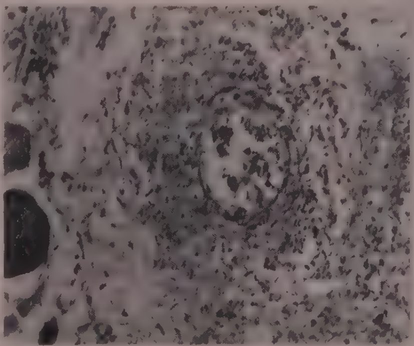

FIG. 2.

Polymorphonuclear infiltration and fibrinoid necrosis of an artery in the skin of a control rabbit at the site of the Arthus reaction. This lesion is similar to that seen in periarteritis nodosa. 200×.

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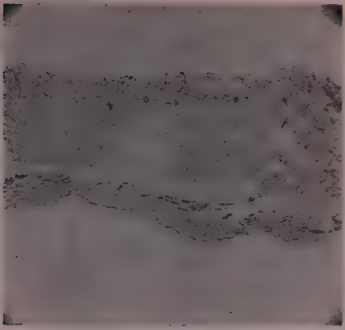

FIG. 3.
Section of skin of a compound E-treated rabbit at site of Arthus reaction. Derma is slightly distended by edema and contains an occasional leucocyte. Note the absence of hemorrhage, necrosis, and vascular lesions. 10×.

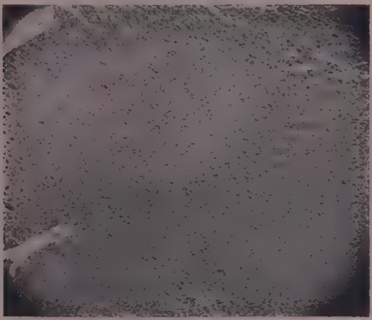

FIG. 4.
Section of thymus of a control rabbit. 33×.

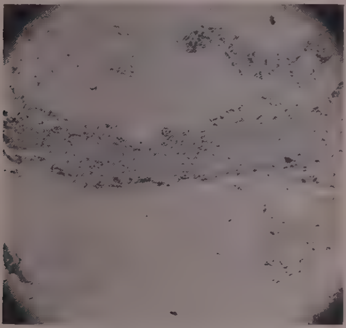

FIG. 5.
Section of thymus of a compound E rabbit. The lymphocytes have almost completely disappeared and only collapsed stroma, blood vessels, and Hassall's bodies remain. 65×.

examination of one of these lesions showed marked leucocytic infiltration of the derma and subcutis. Heart sections from 4 of 6 animals contained minute foci of round cells and occasional polymorphonuclear leucocytes situated in the myocardium. These slight but definite areas of interstitial myocarditis were not seen in any of the sections of the normal, Compound E or ACTH-treated animals.

B. Rabbits injected with egg albumen and treated with Compound E. In contrast to the histological appearance of the skin lesions of the control animals, sections of the small skin lesions of the Compound-E-treated animals showed, at most, slight edema of the derma with few polymorphonuclear leucocytes and round cells scattered here and there. Noteworthy was the absence of the necrotizing arteritis, the hemorrhage, and the necrosis of

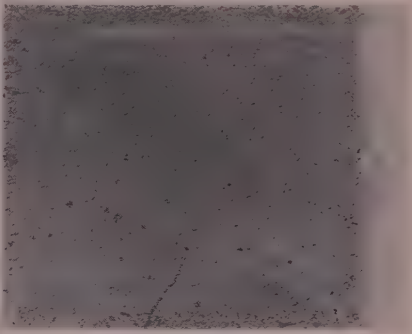

FIG. 6.
Section of spleen of a control rabbit. 18×.

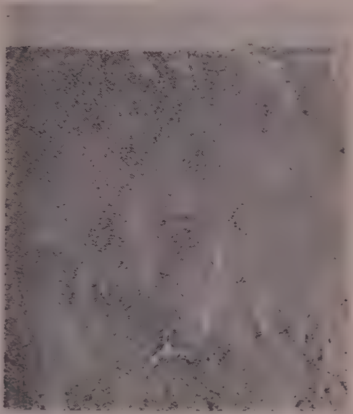

FIG. 7.
Section of atrophic spleen of a compound E rabbit. Note the prominence of the trabeculae and the great number of small malpighian bodies. 18×.

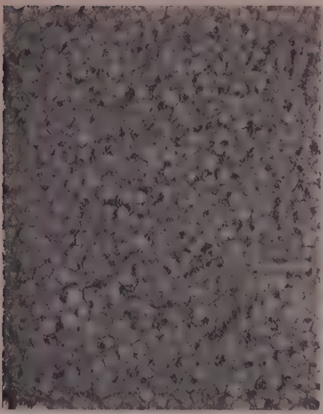

FIG. 8.

Section of liver of a compound E rabbit stained with azure-eosin. The cells are extensively vacuolated and contain little cytoplasm. An occasional hepatic cell appears necrotic. 70×.

subcutaneous muscle, all of which were so prominent in the sections of the skin lesions of 6 of the 8 controls. In all of the Compound E-treated animals the thymus was atrophic. In many instances the lymphocytes had completely disappeared and only the collapsed stroma, blood vessels, and Hassall's bodies remained. As a result of this massive loss of lymphoid elements, the thymuses of the Compound E-treated animals weighed only one-eighth as much as those of the untreated controls (Table I). Like the thymus, the spleens were reduced in size as a result of treatment. They weighed one-third as much as the spleens of the controls. Microscopically, the trabeculae appeared prominent and there was a massive loss of lymphocytes which was reflected by a great increase in the number of small Malphighian bodies occupying a single low-power field.

Compound E had no apparent effect on the size of the mesenteric lymph nodes. However, histologically, germinal centers present in the sections of the nodes from the control animals were absent in the nodes of the animals treated with Compound E. The adrenals of the Compound E animals weighed on the average only one-half as much as the adrenals of the controls but approximately the same as those from 5 normal rabbits. Histologically, the fascicular zones of the cortices appeared to be more narrow than those of the controls. However, lipoid was present in all 3 zones of the cortex. The livers of the Compound E-treated animals presented an extraordinary appearance. In contrast to the purple livers of the control animals, these were gray-yellow in color and approximately three times larger. With azure-eosin stain the hepatic cells appeared greatly enlarged and extensively vacuolated. Small amounts of cytoplasm were present about the nucleus of each cell and, to a lesser extent, just inside the cell membrane. Glycogen and fat stains demonstrated that these otherwise empty cells contained large quantities of glycogen and smaller amounts of fat. Quantitative glycogen determinations demonstrated that, per gram of dried tissue, the livers of the Compound E-treated rabbits contained approximately three times as much glycogen as those of the controls. The livers from 4 control animals contained 34-35 mg of glycogen per g, averaging 63 mg, while those from 4 Compound E-treated rabbits contained as much as 138-233 mg per g averaging 188 mg.

Numerous areas of necrotic cells were encountered in the livers of 5 of the 8 rabbits. The cause of these foci of coagulative necrosis was not apparent. The question, however, arises whether the presence of large quantities of glycogen and fat in the cells might have interfered with their metabolic activities in such a way that they were no longer able to survive. For example, it has been demonstrated that the regenerative capacity of hepatic cells that are overburdened with fat is far less than that of fat-free cells(6). It should be noted that the livers of the animals which had died during the experimental period were only slightly larger than those of the normal and did not contain glycogen. The kidneys were definitely larger than normal. Whether this enlargement was due to the presence of glycogen was not determined. The renal epithelium of the treated animals was no more vacuolated than that of the controls.

Sections of skeletal muscle were removed

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TABLE I. Anatomic Changes in Rabbits Sensitized by Repeated Intracutaneous Injections of Crystalline Egg Albumin and Treated with Compound E or ACTH.

No. of rabbits	Treatment	Avg wt of following organs in g				
		Thymus	Spleen	Adrenals	Liver	Kidneys
8	Sensitized (control)	3.1	2.4	.28	79	15
8	Sensitized and treated with compound E	0.56	0.86	.13	212 (94)*	20
7	Sensitized and treated with ACTH	0.71	1.6	.37	80	16
5	Normal	5.3	1.5	.13	69	17

* The figure in parentheses is the average weight of the livers of the animals which died during the experimental period.

TABLE II. Effect of Compound E and ACTH on Circulating Leucocytes.

Group	Total W.B.C. per cu. mm blood	No. of W.B.C./cu. mm/blood			Normoblasts per 100 leucocytes
		Lymphocytes	Monocytes	Neutrophiles	
Control (avg of 7 animals)	10340	7130	320	2790	120
Compound E (avg of 4 animals)	10190	2230	102	7850	292
ACTH (avg of 6 animals)	9800	5510	153	4140	35

from the right hind leg of 5 animals at a site distant from the local injections of Compound E and egg albumin. These showed numerous small areas of necrotic fibers surrounded by mononuclear cells and an occasional giant cell. The number of circulating lymphocytes was greatly reduced (Table II) by treatment with Compound E. However, there was an increase in the total number of polymorphonuclear leucocytes so that the total white blood counts were unaltered. Numerous nucleated red cells were encountered in the blood, probably as the result of the repeated bleedings. These were somewhat more numerous in the blood of the Compound E-treated animals than in that of the controls. Because of the presence of pseudoeosinophils in the blood of rabbits, accurate eosinophil counts could not be obtained.

C. Rabbits injected with egg albumin and treated with ACTH. The skin lesions of these rabbits varied considerably in severity. Four showed only slight edema and, at least in one case, slight leukocytic infiltration of the derma. Three others showed (a) marked edema, hemorrhage, and neurotropic infiltration of the derma, (b) necrosis of the subcutaneous muscle, and (c) periarteritic lesions of the small arteries. The thymuses of 5 of the 7 animals examined were atrophic. However, lymphocytes were present in greater numbers than in those of the Compound E-treated animals. Two were entirely normal. Although the average weight of the spleens was somewhat less than that of the controls, only two out of seven were definitely atrophic. In contrast to the small adrenals of the Compound E animals, the adrenals of the ACTH animals were large. Grossly, the cortices were unusually thick, but lacking in lipoid. Microscopically the cortex was normal except for the cells of the zona fasciculata which were large, contained abundant granular cytoplasm, and showed almost complete absence of lipoid. The livers from 2 animals contained moderate amounts of glycogen; those from 4 showed slight to moderate fat, and 2 appeared normal. There was also a lymphocytopenia which was not as marked as with compound E. (Table II).

Discussion. The microscopic appearance of the Arthus reactions in the Compound E-treated rabbits differed decidedly from that of the controls. While the skin lesions in the untreated animals showed extensive edema, hemorrhage, and acute inflammation, those from the treated rabbits exhibited only slight edema and leukocytic infiltration. Furthermore, in the skin of 5 of 6 control animals examined, there were extensive vascular changes (consisting of focal fibrinoid degeneration, inflammation and thrombosis of the smaller arteries) similar in all respects to those of periarteritic nodosa. These vascular alterations which are probably the immediate result of antigen-antibody combination were completely absent in the skin of the Compound E-treated animals. From these observations, one might anticipate that Compound E would exert a similar inhibitory effect on allergic periarteritis nodosa. As was obvious from the gross appearance, the skin lesions of the ACTH-treated animals varied considerably in severity although on the average, they were less severe than those of the controls.

In the hearts of 4 of 6 control animals, there were small areas of interstitial myocarditis similar to those which have been described as resulting from hypersensitivity (8). These lesions were not observed in rabbits receiving either Compound E or ACTH. Rich and his colleagues(9) have recently shown that ACTH exerts an inhibitory influence on the development of cardiovascular lesions produced by anaphylactic hypersensitivity to normal horse serum.

Both Compound E and ACTH produced a profound alteration in the lymphoid tissues of rabbits. In the ACTH-treated rabbits there were atrophy of the thymus, occasional atrophy of the spleen and a lymphocytopenia. With Compound E these changes were even more extensive. In a previous study it was shown that Compound E and ACTH inhibit the production of antibody(1). This inhibition of antibody formation was found to be greater with Compound E than with ACTH. Similarly, as shown by the present study, the atrophy of the lymphoid tissues is more marked with Compound E. In view of recent work on the lymphoid origin of antibody(10) one might wonder whether the suppressed antibody formation in rabbits treated with Compound E and ACTH could have resulted from the lymphoid atrophy produced by these two hormones.

The presence of the focal necrosis of skeletal muscle in animals receiving Compound E might well explain the muscular weakness which appeared during treatment. It is interesting to note that excessive doses of this hormone can produce muscular weakness and paralysis in man(11). The Compound E-treated rabbits also showed enlarged livers due to extensive deposition of glycogen and fat. Possibly because of this alteration in fat metabolism the sera became noticeably lipemic after 7 days of treatment. While Compound E produces its effects directly, the effects of ACTH are mediated through the adrenal cortex. This difference in the mode of action of these two hormones was reflected in the size and lipid content of the cortex. With treatment with Compound E the cortex was thin; with ACTH, the cortex was hypertrophic and its fascicular zone was lacking in lipoid.

Summary. 1. The experimental data indicate that Compound E markedly suppresses the pathological alterations of the Arthus reaction ordinarily produced in the rabbit by repeated injections of crystalline egg albumin. The adrenocorticotrophic hormone exerted a decidedly less marked effect. 2. Treatment with Compound E and ACTH produced atrophy of the lymphoid tissues, including the thymus and spleen, and a lymphocytopenia. These alterations were greater with Compound E and in addition, in the animals treated with this hormone, there were extensive deposition of glycogen and fat in the liver, a lipemia and focal necrosis of skeletal muscle. In the animals treated with Compound E the adrenals were small while in those treated with ACTH, the adrenals were enlarged but lacking in lipoid.

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Received November 21, 1950. P.S.E.B.M., 1951, v76.

Paper Chromatography of Blood Plasmas in Multiple Sclerosis.*† (18431)

Roy L. Swank, A. E. Franklin,‡ and J. H. Quastel.
(Introduced by W. G. Penfield.)

From the Department of Neurology and Neurosurgery, McGill University, the Montreal Neurological Institute, and the Montreal General Hospital Research Institute.

Previous observations have indicated the possibility that the degree of severity of multiple sclerosis is related directly to the amount of the daily fat intake(1). This observation has stimulated studies of the effects of high fat meals on the composition of the blood of normal men and of dogs. First, it was shown that 6 to 9 hours following high fat meals the red blood cells and chylomicra in normal humans and dogs have a tendency to aggregate(2). Second, these changes in the physical stability of the blood were found to be accompanied in the dog by alterations in the plasma protein pattern of the paper chromatogram(3).

In the present study it will be shown that with the paper chromatographic technic(4) it is possible to demonstrate significant changes in the plasma proteins of patients with multiple sclerosis.

Material and methods. Plasmas were obtained from a group of intensively studied patients with multiple sclerosis, who were followed at the Neurological Clinic of the Royal Victoria Hospital. All bloods were obtained from fasting patients and were prevented from clotting by heparin sodium salt (Connaught Laboratories). The bloods were obtained at different intervals of time, and when possible, during exacerbations and remissions of the disease. From 1 to 8 tests were made on each patient. All of the 26 patients had well established disease; most of them had been admitted to the Montreal Neurological Institute for diagnosis and treatment; and all but one were ambulatory. Some of the patients were on low fat diets currently being used in this clinic to treat patients with multiple sclerosis. A total of 11 normal subjects was also studied. Three of these were studied at weekly intervals over a period of 6 weeks, and as a control for those multiple sclerosis patients receiving the low fat diet, these 3 subjects were put on the same low fat diet for a period of 3 weeks. The capillary ascent method was used in chromatographic analysis(5). Whatman No. 1 filter paper was used exclusively in this work. Seven sets of conditions were used to examine the plasma of each normal and each patient. One condition did not include the surface active agent, and the others used 0.02, 0.04, and 0.06 ml Tween 81, and 0.01, 0.03 and 0.05 ml Tween 85 per 0.5 ml of the test plasma.§ The illustrated chromatograms show increasing concentrations of Tween 81 in the top row and Tween 85 in the bottom row. Mixing is easier if the surface active agent is placed in a small culture test tube first, then 0.5 ml of plasma is added and these are thoroughly stirred with a glass rod. Then 0.02 ml of 0.3% hemin is added, and aliquots of the resulting plasma solution (0.02 ml) are placed in the lower left hand corner of 8″ x 8″ Whatman No. 1 filter paper. This is allowed to dry, and the filter paper is stapled in the form of a cylinder, taking care to keep the edges from touching. The cylin-

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† We wish to acknowledge the technical assistance of Mrs. Mary Hersey and Miss Rose Mamelak.

‡ Canada Packers Research Fellow, McGill University.

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§ We are indebted to the Atlas Powder Co., Wilmington, Del. (per G. F. Sterne & Sons, Brantford, Ont.) for samples of Tween 81 (Polyoxyethylene sorbitan monooleate) and Tween 85 (Polyoxyethylene sorbitan trioleate).

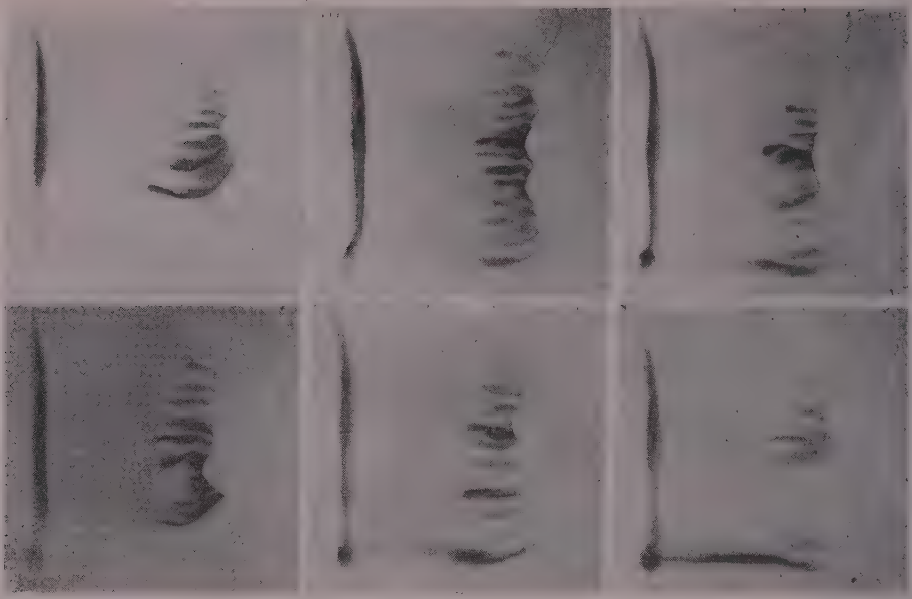

FIG. 1.

Shows 1 chromatogram of a series of 6 from a normal woman 33 years of age. The illustrated chromatograms show increasing concentrations of Tween 81 (.02, .04, and .06 ml per .5 ml of plasma) in the top row, and increasing concentrations of Tween 85 (.01, .03, and .05 ml per .5 ml of plasma) in the bottom row.

ders are placed standing in a pyrex dish containing 100 ml M/10 sucrose, and covered with a bell jar, and the solvent is allowed to reach the top of the paper. This requires about 1-1½ hours. The cylinders are air-dried, unstapled, and restapled at 90° to the original direction. They are again placed erect in a pyrex dish which contains 100 ml M/10 NaK tartrate, and the solvent allowed to go about two-thirds up the paper. After drying, the papers are streaked with the benzidine reagent, as described previously(4). The chromatograms are photographed after a slight delay (about a minute) to allow full color development, and before the characteristic brown background appears.

Results. Protein patterns observed in healthy, young adults. Fig. 1 shows 1 chromatogram of a series of 6 taken at weekly intervals from a healthy woman 33 years of age. The protein pattern in this subject varied very little from week to week during this period. The maximum variation consisted of an absence of several central fractions in the chromatogram obtained when 0.03 ml Tween 85 was added to the plasma. Fig. 2 shows 1 chromatogram of a series of 6 at weekly intervals from a healthy man 29 years of age. Even greater consistency of the protein pattern was shown in this series of tests. Nine other controls (6 women, 3 men) were examined. They showed similar protein patterns with no great variations upon repeated testing. When 0.05 ml Tween 85 was used with the plasma, few protein fractions were observed in either the control or experimental subjects.

The chromatograms of normal, healthy persons appear to vary to some extent from time to time. The variations, however, the causes for which are as yet unknown, have not affected the general protein pattern as observed in normal individuals. Generally speaking the normal pattern indicates the presence of a large number of fractions in all conditions of examination with the exception of the higher concentrations of surface active agents (see Fig. 1-2). It is possible

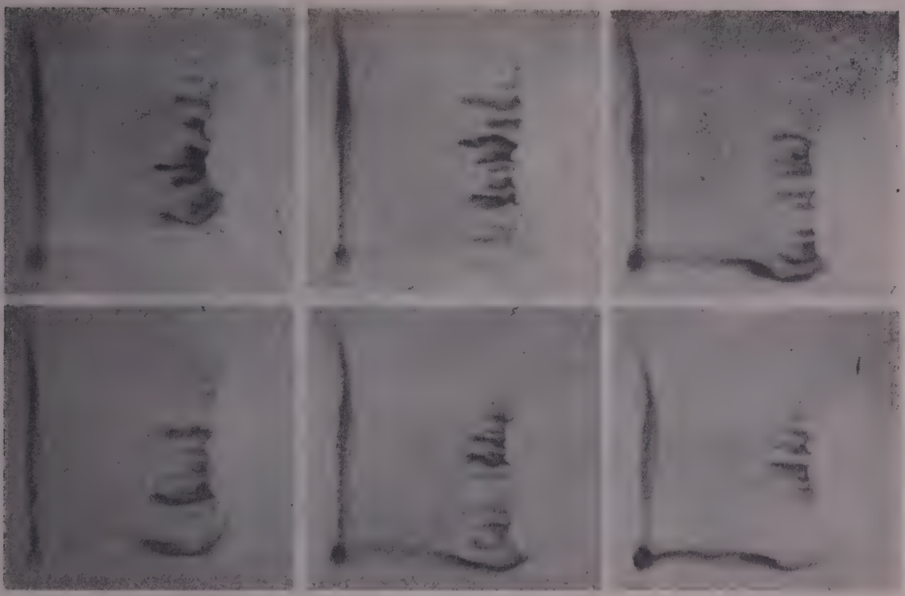

FIG. 2.
Shows 1 chromatogram of a series of 6 from a normal male 29 years of age.

that in normal women these variations are greater than in men, but insufficient material prevents any conclusions on this point. The relationship of the menstrual cycle to the plasma proteins may be of importance in the apparent greater variation observed in women, but this has not been studied.

Protein patterns observed in multiple sclerosis patients. Of a total of 81 chromatograms from 26 patients with multiple sclerosis, 31 tests in 17 patients showed distinct abnormalities, and 1 or more tests in each of 4 patients indicated probable abnormalities. In 5 patients all the chromatograms were normal, despite the occurrence in 2 of them of mild exacerbations of disease during the period of study. In one of the latter 2 patients the chromatograms were carried out 4 days after, and in another case one day after the onset of mild retrobulbar neuritis (as evidenced by a sudden partial loss of vision with return to former vision in about 2 weeks).

In 3 patients striking changes were observed in the chromatogram during exacerbations of disease. These changes were preceded by normal (2 cases), or nearly normal (1 case) chromatograms, and were followed by a return of the chromatogram towards (or to) normal during the subsequent clinical remissions. These patients were not on a low fat diet when the chromatograms before and during the exacerbations were carried out. During the period of remission, however, they were given the low fat diet. An example of the changes in the chromatograms in one patient is shown in Fig. 3, 4, and 5. The chromatogram prior to exacerbation (Fig. 3) was essentially normal. Five to 6 days after the onset of the exacerbation, and while the neurological signs were still increasing, the chromatogram (Fig. 4) showed an absence both of a large number of central fractions, and of some of the upper fractions (compare with Fig. 1-2). Eight days later the protein pattern was little altered. During clinical remission, and 11 weeks (Fig. 5) later, the chromatogram returned towards normal.

Another example of the protein pattern changes during an exacerbation is shown in Fig. 6-8. It will be noted that many of the upper fractions are absent during exacerbation

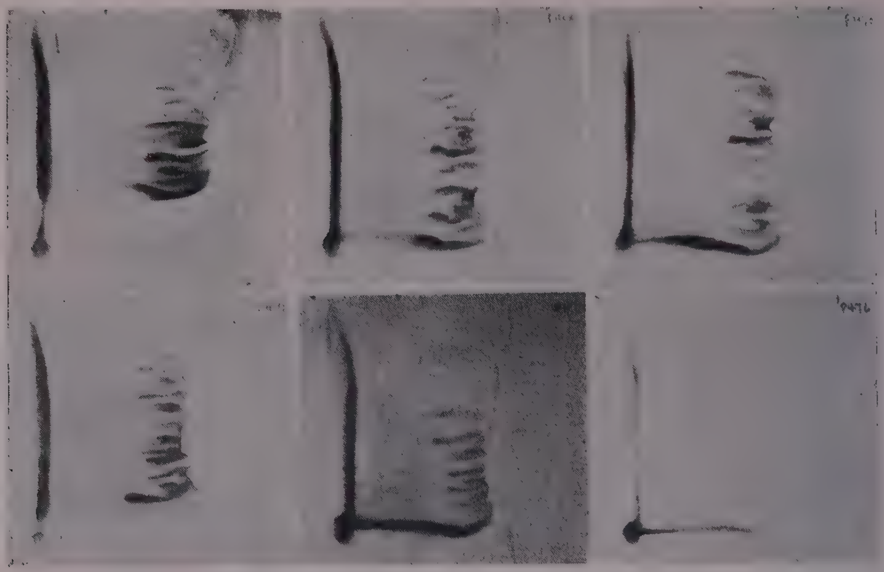

FIG. 3.

Fig. 3, 4, and 5 show a series of chromatograms of a male patient with multiple sclerosis. The chromatogram in Fig. 3 was made from plasma obtained April 6, just before an exacerbation. Fig. 4 shows a chromatogram during the exacerbation (April 13), and Fig. 5 (June 26) shows a chromatogram during remission.

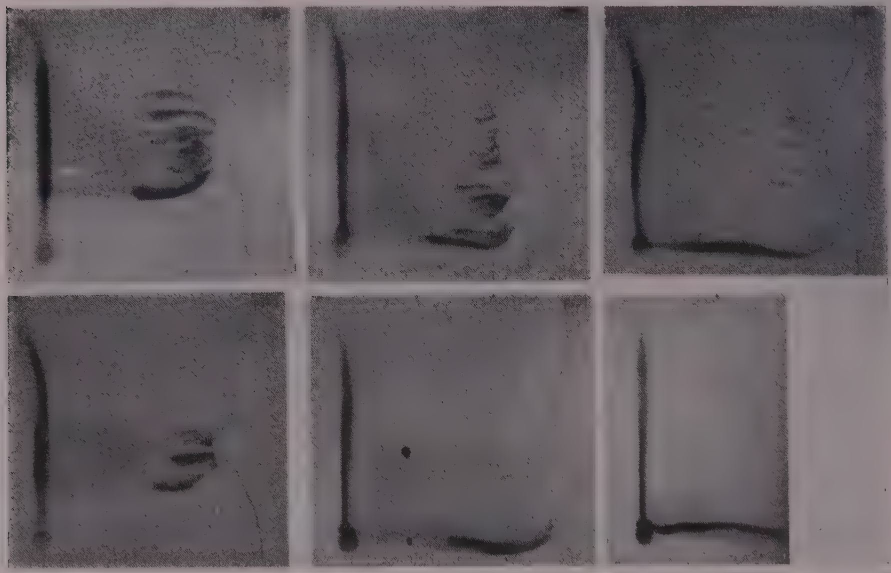

FIG. 4.

(Fig. 7) and that these gradually reappear in the subsequent chromatograms (see Fig. 8; 5 weeks later).

Eleven other patients, without clinical evidence of exacerbation of disease, exhibited changes which were considered distinctly ab-

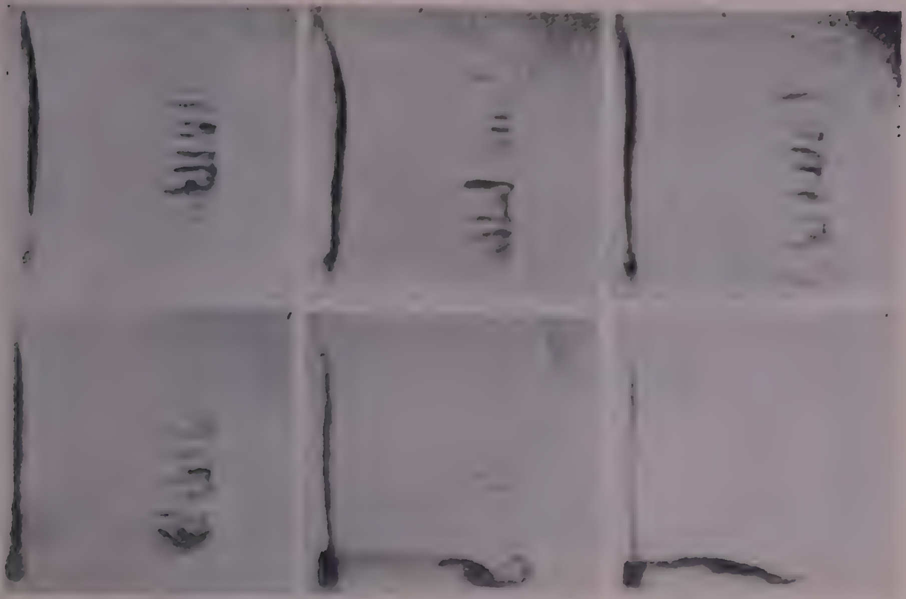

FIG. 5.

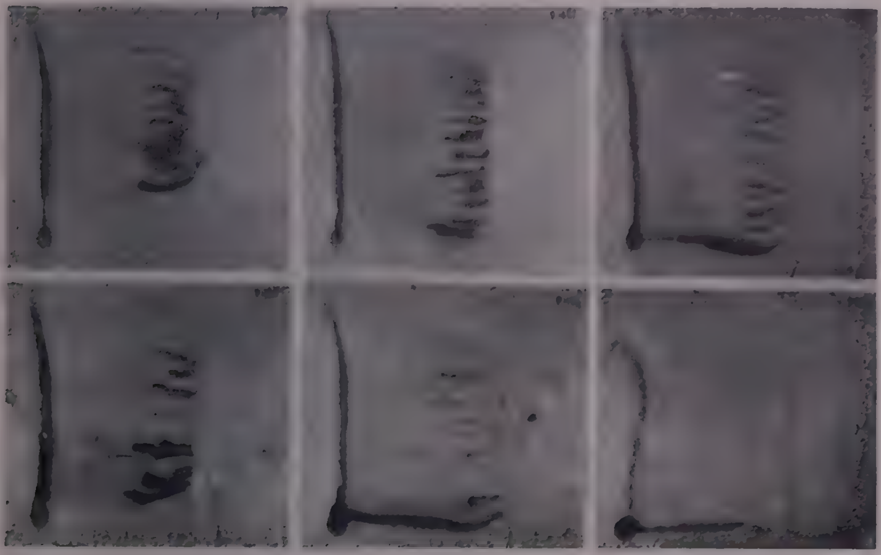

FIG. 6.

Fig. 6, 7, and 8 show a series of chromatograms of a male patient with multiple sclerosis. Fig. 6 was chromatographed (April 6) prior to an exacerbation. Fig. 7 shows the chromatograms 3 days after the onset of an exacerbation (April 14) and Fig. 8 (May 18) shows the chromatogram during remission.

normal, in one or more chromatograms. All of these patients suffered from a relatively active disease as judged by the occurrence of frequent and severe exacerbations during the preceding years. Three additional patients with steadily progressive disability consisting

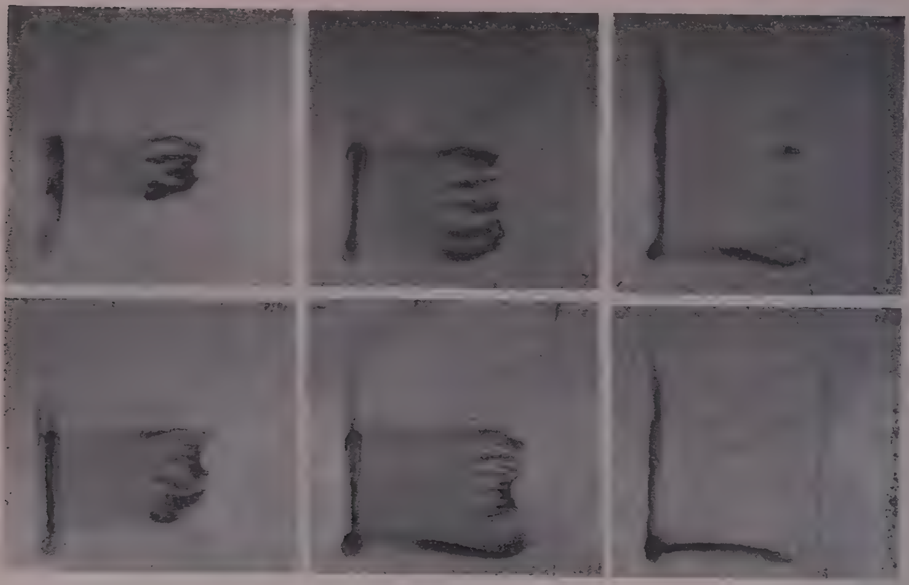

FIG. 7.

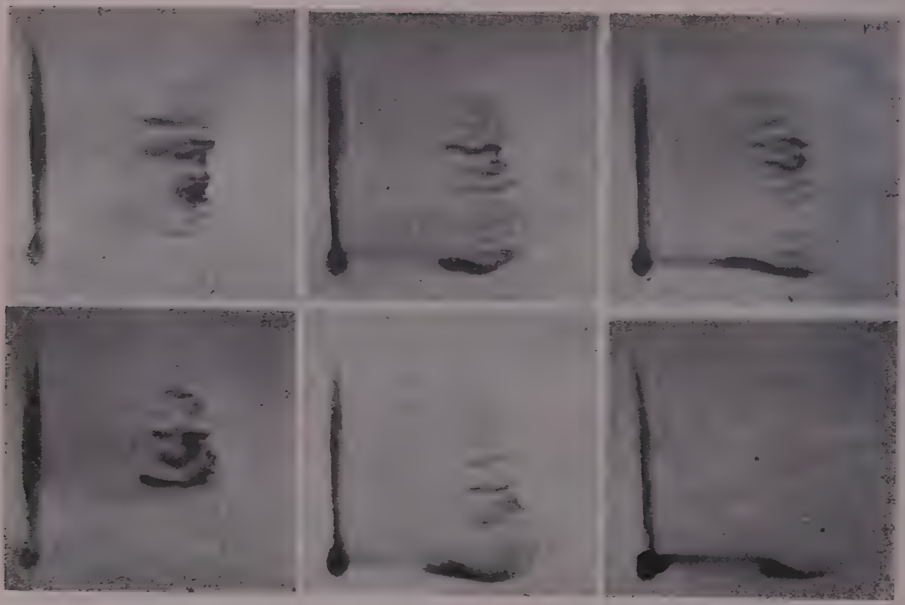

FIG. 8.

of mixed ataxia and spasticity displayed definite alterations of the protein pattern in the chromatograms (see *e.g.* Fig. 9).

Effects of blood transfusions. Preliminary experiments have indicated that, following transfusion, the protein chromatogram tends to revert to normal. This matter is being investigated.

Discussion. The significance of the changes which have been observed in the

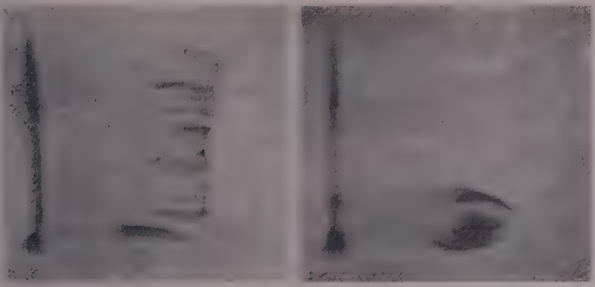

FIG. 9.
Chromatogram of a patient with steadily progressive symptoms of multiple sclerosis. Only the 0.02 ml Tween 81 (left hand side) and 0.01 Tween 85 (right hand side) conditions were used.

plasma proteins of patients with multiple sclerosis is not known. The limited observations suggest that these changes usually do not precede a clinical exacerbation of the disease by an appreciable interval of time. It is difficult to assess the time interval, however, because the exact time of the onset of an exacerbation cannot be established with certainty. Neither do the changes in the protein pattern continue to be present for many weeks after the onset of the exacerbation. If the pattern of plasma proteins shown by paper chromatography is secondary to, and determined by the amount of nervous tissue undergoing change, then it is reasonable to assume that small lesions (for example mild retrobulbar neuritis) might not alter the protein pattern. On the other hand, extensive lesions might occur in so-called silent areas of the brain and produce marked changes in the chromatograms, and yet be unaccompanied by symptoms.

The chromatograms may reflect changes in the blood which occur concomitantly with, or even prior to, the development of the lesions in the nervous system. It may also be considered that the proteins might be modified by the chemical changes attending the occurrence of these lesions.

The abnormalities in the plasma protein chromatograms do not appear to be specific for multiple sclerosis since very abnormal patterns have also been observed in multiple myeloma and liver cirrhosis.¶

Unpublished observations by one of us (R.L.S.) have shown that sedimentation rates of patients with multiple sclerosis (determined at weekly or monthly intervals) are frequently abnormally high, but the evidence so far has not established a clear correlation between such changed sedimentation rates and the abnormal chromatograms shown by these patients. No attempt was made to correlate the plasma protein patterns with the Lange reaction in the spinal fluid.

Summary. Using the paper chromatographic technic significant changes in the protein patterns have been demonstrated in patients with multiple sclerosis. We are unable to state at present the relationship of these changes to the activity of the disease, but our evidence would seem to indicate that they may occur concomitantly with, or immediately following the onset of exacerbation. Furthermore the changes in protein patterns revert to normal during remissions.

¶ Unpublished observations by Franklin and Quastel.

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Relation between Certain Myeloma Proteins and Normal Gamma Globulin (18432)

H. G. Kunkel, R. J. Slater, and R. A. Good.
From the Hospital of the Rockefeller Institute for Medical Research, New York City.

The recent purification of human gamma globulin by alcohol fractionation has led to studies of its immunological properties(1-3). The work has been hampered by the fact that the various gamma globulin preparations are not single proteins. Two materials, called gamma$_1$ globulin and gamma$_2$ globulin, have been separated that differ electrophoretically; however, each of these remains nonhomogeneous. Although each absorbs all of the antibody against the total normal gamma globulin, Kabat and Murray(2) have demonstrated immunological differences between these two preparations. The recent observations of Cohn, Deutsch and Wetter have shown clearly that the gamma globulin fractions contain more than one antigen. The present study represents an attempt to clarify the relationship between the fractions of gamma globulin through a study of the special readily purified proteins that exist in tremendous quantity in the serum of patients with multiple myeloma. Evidence was obtained for an immunological similarity between various myeloma proteins and a portion of normal human gamma globulin.

Two preparations of human gamma globulin (II-1,2 and II-3), obtained from the laboratory of Dr. E. J. Cohn, were injected into rabbits in the alum precipitated form according to the usual schedule(4). Good antisera were obtained in approximately 40% of the animals. This antiserum did not give a precipitin reaction with human albumin. An attempt was made to estimate the gamma globulin concentration in various sera by employing quantitative precipitin studies in the region of extreme antibody excess. A technic was used similar to that previously described for albumin(4). In confirmation of the work by Jager and coworkers(1), consistently higher values were obtained for gamma globulin by this procedure than by electrophoretic analysis. Values for normal serum ranged between 1.4 and 2.2 g/100 cc with several different antisera. The serum of patients with nephrosis, known to contain very small amounts of gamma globulin, gave very low values by the immunological method; the serum of patients with cirrhosis, containing large amounts of gamma globulin as determined electrophoretically, gave values higher than the normal range. There was a general correlation, although not a close one, between the immunological and the electrophoretic values for gamma globulin.

The sera of 5 different patients with multiple myeloma were also studied in a quantitative manner with the gamma globulin antisera. Each of these sera contained a very high total protein due to a single sharp electrophoretic peak which varied in mobility in the different sera (Table I). Fig. 1 shows the quantitative precipitin curves for 2 normal sera and myeloma serum D. It is apparent that the myeloma serum demonstrates an equivalence zone shifted to the

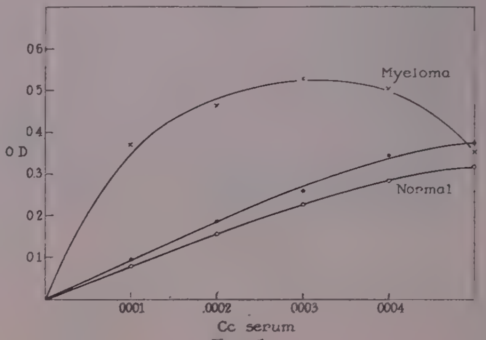

FIG. 1.

Quantitative precipitin curves showing the intense precipitin reaction given by myeloma serum D as compared to 2 normal sera. O.D. refers to ninhydrin color of the antigen-antibody precipitate.

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TABLE I. Electrophoretic Concentration and Mobility of the Myeloma Protein in the Serum of 5 Patients with Multiple Myeloma.*

	Total protein, g/100 cc	Myeloma protein, g/100 cc	Mobility $U \times 10^5$
Myeloma D	13.5	10.20	2.15
” Y	17.2	13.70	1.20
” X	11.8	8.75	1.11
” S	11.0	6.13	2.15
” L	13.4	9.20	3.30

* Barbital buffer, pH 8.6, μ 0.1, descending boundaries.

left into the region of antibody excess relative to normal serum. This was also true of myeloma sera, Y, X, and S. However, serum S showed a somewhat different reaction. The quantitative curve showed a small but definite peak in the region of extreme antibody excess at dilutions where normal serum failed to give any reaction and where the other myeloma sera had given a more intense reaction. Similar results were obtained with 3 different antisera. It was possible to obtain a quantitative value for gamma globulin in each of these sera that approximated the high concentration of the abnormal myeloma component. Since each of these sera contained only a single electrophoretic component that was present in a concentration greater than normal, the only possible explanation for the immunological data was that the special myeloma protein reacted with the gamma globulin antisera. Myeloma serum L, despite a very high total protein, gave a curve resembling that of normal serum but with an even less intense precipitin reaction at low dilutions.

Quantitative precipitin studies of purified myeloma proteins and gamma globulin antiserum. The special myeloma proteins from each of the 5 sera were obtained in varying degrees of purity. Myeloma Y was obtained by ethanol fractionation in the cold; a procedure similar to that described by Deutsch and coworkers(5) was employed. The final preparation when tested electrophoretically showed a single sharp component at pH 8.6 that made up 96% of the total protein. It was not tested under other conditions or in the ultracentrifuge. The purified protein, myeloma X, was furnished by Dr. Mary L. Petermann and Dr. G. E. Cogin of the Sloan-Kettering Institute for Cancer Research. It was isolated from myeloma serum by ethanol and salt fractionation. Their data indicate that the purified protein was 98% one component electrophoretically and was homogeneous at ionic strength 0.1 between pH 4.0 and 10.0. Ultracentrifugal analysis showed 95% of S = 7 component and 5% of S = 10 component. The remaining myeloma proteins were purified by electrophoretic separation.

Fig. 2 illustrates the quantitative precipitin curves for myeloma Y and X as compared with II-3 gamma globulin when studied with II-3 antiserum. It is apparent that each of the myeloma proteins gives a definite precipitin reaction with the gamma globulin antiserum. However, the intensity of the reaction is not so great as for the normal gamma globulin. Myeloma Y gives a somewhat stronger reaction than myeloma X. Myeloma D, which is not illustrated in the chart, gave a curve almost identical with that for myeloma Y despite a distinct difference in electrophoretic mobility. It appeared as though the myeloma proteins reacted with only a portion of the total antibodies to normal gamma globulin. This was verified by absorption experiments. Antiserum against II-3 gamma globulin was carefully absorbed over a period of 6 days with progressively increasing amounts of myeloma Y until no further precipitate was obtained. This absorbed antiserum still gave a precipitin reaction with II-3 gamma globulin. The curves for II-3 gamma globulin with the whole antiserum and with that absorbed with myeloma Y are compared in Fig. 2. Myeloma X and D gave no precipitin reaction with the Y absorbed antiserum. The II-3 antiserum was also absorbed with myeloma X. This absorbed antiserum gave a strong reaction with II-3 gamma globulin and also with myeloma Y. It was apparent that although myeloma Y removed all of the antibodies to myeloma X, the reverse was not true. This was to be expected from the slightly different quantitative precipitin curves. Myeloma D absorbed all of the antibody to myeloma Y and X. Similar results were obtained by absorption of II-1,2

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5. Deutsch, H. F., Alberty, R. A., and Gosting, L. J., *J. Biol. Chem.*, 1946, v165, 21.

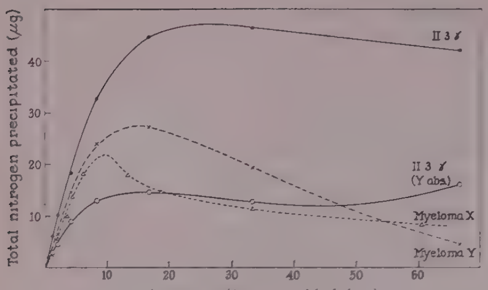

FIG. 2.

Quantitative precipitin curves of II-3 γ globulin, myeloma Y and myeloma X with antiserum to II-3 γ. The curve for II-3 γ with II-3 γ antiserum absorbed with myeloma Y is also illustrated.

antiserum. Purified myeloma protein L gave no reaction with gamma globulin antisera.

Reaction with antiserum to purified myeloma protein. Antiserum to myeloma X was obtained by employing the adjuvant technic of Freund. Previous attempts with alum precipitated myeloma proteins had failed to produce antibodies. A potent antiserum was obtained which, in addition to giving a precipitin reaction with myeloma X, also gave a strong reaction with II-1,2 gamma globulin as well as with normal serum. When quantitative precipitin curves were obtained, it was found that the II-1,2 gamma globulin gave a lower curve with this antiserum than did myeloma X protein when comparisons were made in terms of mg of nitrogen. Neither curve fell to the baseline in the region of antigen excess. It appeared that the II-1,2 gamma globulin contained protein which did not react with the myeloma antiserum. These observations correlated with those obtained with II-1,2 antiserum where both the quantitative precipitin curves and the absorption experiments indicated that the gamma globulin antiserum contained further antibodies in addition to those reacting with the myeloma protein. Absorption experiments with the myeloma antiserum indicated that the antibodies were entirely absorbed with the myeloma protein and no further precipitate was obtained with the II-1,2 gamma globulin. The latter also removed all of the antibodies to the myeloma protein. Despite the fact that some evidence was obtained indicating that the myeloma antiserum contained antibody to more than a single antigen, it definitely lacked certain of the antibody components found in the gamma globulin antiserum.

Discussion. The immunological data offer strong evidence for an antigenic relationship between the different myeloma proteins despite differences in electrophoretic mobility. Of particular interest is the fact that two of the myeloma proteins, D and Y, showed identical quantitative precipitin curves and each absorbed all of the antibody to the other, although one showed an electrophoretic mobility approximately twice that of the other. However, the myeloma protein showing the most rapid mobility of the 5 studied differed most markedly from the other proteins and gave no precipitin reaction with normal gamma globulin antiserum. Previous immunological studies with the proteins appearing in multiple myeloma have dealt chiefly with the Bence-Jones protein in the urine. This protein differs greatly in physical and chemical properties from normal serum proteins and from the myeloma proteins discussed in the present report and it has been found to produce a specific antiserum that does not react with normal proteins. Recently, Wuhrmann *et al.*(6) have reported that a myeloma protein showing a rapid electrophoretic mobility produced a specific antiserum that did not react with normal serum components. Myeloma L is possibly similar to that described by Wuhrmann as it failed to react with either normal gamma globulin antiserum or with myeloma X antiserum. Also, it showed a mobility similar to that of Wuhrmann's myeloma protein. It appears that the greater the mobility of the myeloma protein, the further removed antigenically it is from normal gamma globulin, as in myeloma L.

Both the precipitin curves and the absorption experiments indicate that the majority of myeloma proteins are immunologically related to a portion or normal gamma globulin. At least in the case of antisera to two different preparations of gamma globulin,

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6. Wuhrmann, Von F., Wunderley, C., Hässig, A., and Hugentabler, F., *Helv. Med. Acta.*, 1949, v16, 279

similar results were obtained and the myeloma proteins absorbed a sizeable amount of the total antibody. This interpretation was confirmed by the reaction obtained between normal gamma globulin and myeloma X antiserum. Through the medium of absorption of gamma globulin antiserum with purified myeloma proteins, a method was obtained for dividing the normal gamma globulin antiserum into two portions, a myeloma related portion and a non-myeloma related portion. Studies are under way in which these two antisera are being used. Preliminary observations have indicated that the increased gamma globulin found in the serum of patients with liver disease is of the myeloma related type. Further subdivision of the gamma globulin antiserum was possible by absorption with different myeloma proteins.

Since the myeloma proteins are found in the serum in huge concentrations in a disease characterized by great proliferation of plasma cells, it would appear that these cells are involved in the production of these proteins. Therefore, the results obtained showing a relationship between myeloma proteins and a portion of normal gamma globulin adds further evidence for a role by plasma cells in the production of at least a component of normal gamma globulin.

Summary. 1. Antisera against two preparations of normal gamma globulin reacted quantitatively with the special myeloma protein found in the serum of 4 patients with multiple myeloma. 2. The myeloma protein from the serum of a fifth patient showing the most rapid electrophoretic mobility failed to react with gamma globulin antisera. 3. Absorption of gamma globulin antiserum with purified myeloma proteins removed only a portion of the total antiserum, thus dividing the antibody into fractions. 4. Antiserum prepared against a highly purified preparation of a $gamma_2$ myeloma protein gave a precipitin reaction with normal gamma globulin.

The authors are indebted to Dr. Mary L. Petermann for furnishing two of the myeloma sera in addition to a purified preparation of myeloma protein and to Dr. Merrill W. Chase for furnishing adjuvants for immunization.

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Adsorption of Bovine Alkaline Phosphatase on Diatomaceous Silica. (18433)

Charles A. Zittle and Edward S. DellaMonica.
(Introduced by G. W. Irving, Jr.)
From the Eastern Regional Research Laboratory, Philadelphia, Pa.*

Recent reports have described the adsorption of enzyme-carrying cell particles(1,2) and viruses(3,4) on various types of Celite (diatomaceous silica) from salt-containing solutions. Similarly, when salts are present, a partially purified alkaline phosphatase of cow's milk(5) is strongly adsorbed on the Celite Filter-Cel. The adsorption affords some increase in the purification of the enzyme, since the non-phosphatase protein is less strongly adsorbed. The results, however, are of more interest in explaining the adsorption of certain complex proteins on Celite. A comparison of adsorption on various Celites suggested that the presence of clay in its natural state was necessary for adsorption. The influence of various physical and chemical factors on the adsorption was also investigated.

Materials and methods. The preparation of the alkaline phosphatase of cow's milk has been described(5). Since solutions of the phosphatase were somewhat unstable, the preparation was dried from the frozen state. The assays were performed in ethanolamine buffer as described(5). Enzyme activities are expressed as colorimeter readings, a reading of 1 being taken as 1 unit. The activity of the dry preparation was 225 units/mg. This preparation,[†] which is strongly adsorbed on Filter-Cel, was used in all the experiments. The adsorptions were performed in 0.1 M buffers: collidine, pH 7.0(6); tris-(hydroxymethyl)-aminomethane, pH 8.0(6); and ethanolamine, pH 9.6(5). Salts, principally NaCl and Na_2SO_4, and the dry milk phosphatase were added to the buffers. Filter-Cel was added to 10.0 cc of the enzyme-salt-buffer mixture. After stirring for 2 minutes, which was adequate for maximum adsorption, the mixture was centrifuged, and the supernatant solution was decanted and assayed for enzyme activity, together with the original enzyme-salt-buffer solution. In the elution experiments, 10.0 cc of eluent were added to the sedimented adsorbent, stirred for 2 minutes, and centrifuged, and the supernatant solution was assayed for activity.

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* One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

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Results. The effect of varying the amount of Filter-Cel with several concentrations of the milk phosphatase in 1.5 M NaCl, pH 8.0, at 23°, is shown in Fig. 1. The increase in adsorption of the phosphatase on dilution, with small amounts of Filter-Cel, as well as the shape of the curves, is characteristic of most adsorptions. The limit of adsorption, however, of about 80% for each dilution of phosphatase is unusual and suggests that an unadsorbed phosphatase is present to the extent of 20%.

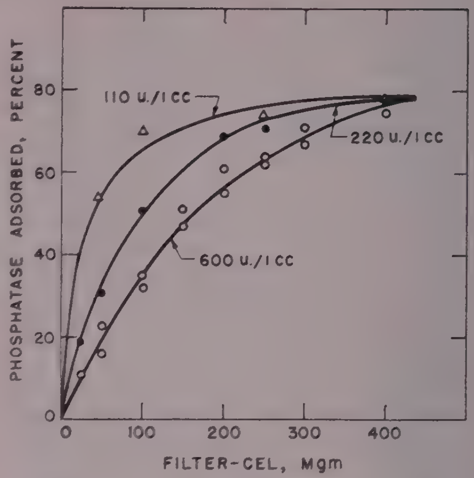

Fig. 1.

Adsorption of milk phosphatase on different amounts of Filter-Cel with several concentrations of the enzyme from 1.5 M NaCl, pH 8.0, at 23°.

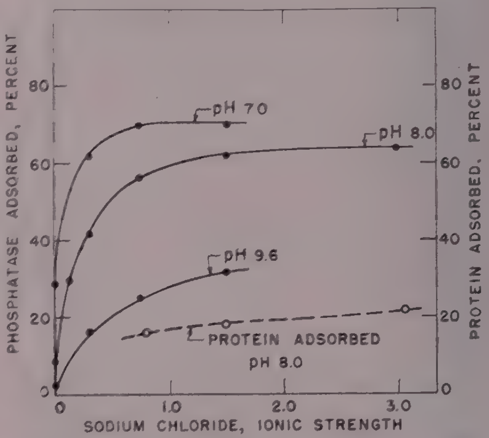

Fig. 2.

Effect of salt concentration and pH on adsorption of milk phosphatase on 250 mg Filter-Cel.

The influence of NaCl concentration and pH on the adsorption of the milk phosphatase on 250 mg of Filter-Cel is shown in Fig. 2. Although the results at each pH were obtained with a different buffer, the regular variation with pH suggests there were no specific effects attributable to the buffer compound. The percentage of the total protein adsorbed with several concentrations of NaCl at pH 8.0 is shown in Fig. 2 also. It is evident that if the enzyme could be totally eluted from the Filter-Cel, 3-fold purification of the phosphatase could be obtained in one treatment with Filter-Cel. The phosphatase concentration in these experiments was 600 u/cc.

[†] Solutions of this phosphatase preparation are opalescent. Further purification has given clear solutions that are poorly adsorbed on Filter-Cel. These and other results suggest that the phosphatase adsorbed on Filter-Cel is a complex, perhaps lipid-containing. In the course of further purification in which treatment with lipase is employed, the complex is dissociated to give a phosphatase not adsorbed on Filter-Cel. The small amount of phosphatase not adsorbed on Filter-Cel from the preparation used in the present experiments may indicate the presence of the dissociated phosphatase. The mere presence of non-phosphatase protein would by interference be more likely to reduce rather than to increase adsorption of phosphatase.

6. Gomori, G., Proc. Soc. Exp. Biol. and Med., 1946, v62, 33.

Although the shape of each adsorption curve suggests an equilibrium between adsorbent and enzyme, elution of the enzyme activity never exceeded about 60%, with a consequent increase in purity of only 1.5- to 2-fold. When adsorbed on Filter-Cel from salt-containing buffers, none of the phosphatase could be eluted at the same pH even with salt-free buffer. However, the enzyme could be partly eluted by increasing the pH, for example, enzyme (600 u/cc) adsorbed on 250 mg of Filter-Cel at pH 8.0 from 1.5 M NaCl could be eluted to the extent of 37% with salt-free ethanolamine buffer at pH 9.6. The recovery was related to the amount of adsorbent; 17.5, 65 and 67%, respectively, were recovered from 500, 100 and 50 mg of Filter-Cel. Calculations showed that the non-recovered enzyme was proportional to the amount of Filter-Cel, namely, about 65 u/100 mg. This suggests that there is a firmly held portion of enzyme that cannot be eluted and a portion of enzyme adsorbed thereon or on less reactive sites that can be eluted. The amount that can be eluted is increased when the ratio of enzyme to adsorbent is increased. This was also evident when 250 mg of Filter-Cel on which phosphatase had been adsorbed from 0.8 M Na_2SO_4, pH 8.0, and then washed with ethanolamine buffer, was used as adsorbent. Only 36% of phosphatase was adsorbed in this case, but 82.4% of it was eluted with ethanolamine buffer.

The recovery of phosphatase was greater when Na_2SO_4 was used instead of NaCl at the same ionic strength. With 250 mg of Filter-Cel, the recovery of enzyme adsorbed from Na_2SO_4 was 50% as compared with 37% from NaCl. Tests in which Na caprylate, Na citrate, NaK tartrate or K thiocyanate replaced the Na_2SO_4 led to no improvement in the elution. Adsorption increased with rise in temperature (5° to 37°), but variation of this factor in the elution offered no advantage. When tested in 250-mg portions at pH 8.0 with Na_2SO_4 present, the following adsorbents adsorbed the phosphatase only slightly (0 to 14%); Alumina (80-200 mesh), talc, α-cellulose, filter paper, Norite, calcium phosphate, and Lloyd's reagent. Bentonite gave a strong adsorption (72% from 0.4 M Na_2SO_4). The phosphatase was not eluted with ethanolamine buffer, but it was eluted with 0.1 M collidine to the extent of 53%. Interesting results were obtained with Celites other than Filter-Cel. At pH 8.0, with 1.5 M NaCl and 250 mg of the Celite, Standard Super-Cel adsorbed 12% of the phosphatase, and Hyflo Super-Cel only 3% as compared with 62% for Filter-Cel. Data supplied by the manufacturer show that Filter-Cel, and Standard and Hyflo Super-Cels have very much the same particle size. Particle size, therefore, cannot account for the differences in adsorption noted. These Celites, however, differ in other respects. Filter-Cel is a natural product, whereas Standard Super-Cel is calcined, and Hyflo Super-Cel is calcined after a flux has been added. It has been reported(7) that calcination converts the hydrated colloidal clay, present in Celite to the extent of about 4% (as Al_2O_3), to the less adsorbent anhydrous aluminum silicate. This suggests that the adsorption of the milk phosphatase shown by Filter-Cel may be due largely to its content of clay.

Discussion. The effect of increasing ionic strength on the absorption of milk phosphatase parallels the results of Shepard and Tiselius(8) for the adsorption of serum albumin on silica gel. The studies are alike also in showing a decrease in adsorption at higher pH values.

Irreversibility of adsorption, noted in the

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experiments reported here, has also been noted by Mitchell *et al.*(9) in studies of amylase, adenosine deaminase and "adenylic acid phosphatase" in Taka-diastase on their filter paper chromatopile. In an initial run with a $(NH_4)_2SO_4$ gradient at pH 6.5, the enzymes were separated, the "phosphatase" being least adsorbed(9). In second runs with samples from activity peaks, however, the enzyme failed to move. Presumably a strongly adsorbing component of the Taka-diastase was acting as a displacing agent in the first run. The enzymes could be made to move in the second runs by raising the pH to higher levels. Runs at a pH of 6.5 and constant $(NH_4)_2SO_4$ concentrations gave a wide spread of the enzyme activity, with a frontal peak in most cases. Such results would follow from the present observations on two types of adsorption, that firmly held and that which can be eluted, the latter perhaps being held on the former.

The adsorption of the milk phosphatase on various Celites is roughly paralleled by the adsorption of certain viruses on Celites, suggesting that with viruses also the clay in the Celites might be the active adsorbent. The pneumonia virus of mice, for example, is strongly adsorbed at pH 6.8 from 0.15 M NaCl on Filter-Cel, Celite no. 505, and Super-Cel, less strongly on Hyflo Super-Cel and Celite no. 503(4). Here also elutions were successful only at high pH values, pH 9 and above.

Summary. The adsorption of a preparation of bovine alkaline phosphatase on Filter-Cel was investigated in respect to amount of adsorbent, concentration and kind of salt, pH and temperature. Calcined Celites were poor adsorbents for the phosphatase.

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Effects of Vitamin B_{12} on Diethylstilbestrol and Thyroprotein Action in Male Rats.*† (18434)

Joseph Meites and J. C. Shay.

From the Dept. of Physiology and Pharmacology, Michigan State College, East Lansing, Mich.

Recently vit. B_{12} has been demonstrated to counteract the inhibition of growth induced by feeding large doses of thyroid-active substances(1-4) or thiouracil(5) to young rats. It was considered possible that vit. B_{12} might likewise counteract the inhibition of growth which has been observed in young rats treated with diethylstilbestrol(6). It was also of interest to determine whether the effectiveness of vit. B_{12} in these respects could be explained by an increase in food consumption and/or an increase in the catabolism or rate of excretion of the hormones administered.

Methods. Immature male albino rats (Carworth strain) were divided into 10 uniform groups of 10 each, and were fed the following basal ration for 20 days: yellow corn meal, 35%; ground wheat, 25%; linseed oil meal, 10%; whole milk powder, 20%; alfalfa leaf meal, 6%; brewers' yeast, 3%; and table salt, 1%. Diethylstilbestrol‡ or thyroprotein‡ were mixed into the ration in amounts of 0.10 and 0.16 per cent respectively. Vit.

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† An abstract of part of the data presented in this paper appeared in *Fed. Proc.*, 1950, v9, 87.

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TABLE I. Effects of Vitamin B_{12} on Diethylstilbestrol and Thyroprotein Action in Male Rats.

Group	Treatment	Avg body wt Orig., g	Avg body wt Final, g	Avg total gain in body wt, g	Avg daily food intake, g	Avg testis wt Actual, mg	Avg testis wt Per 100 g body wt, mg	Avg sem. ves. wt Actual, mg	Avg sem. ves. wt Per 100 g body wt, mg
1	Control	55.0	115.6	60.6	9.6	1207.6	1016.3 ± 94.7*	48.2	41.5 ±4.1*
2	.2 μg B_{12}	58.6	120.8	62.2	9.5	1011.7	834.1 ± 99.5		
3	.1% stilb.†	55.0	80.9	25.9	6.0	272.4	331.3 ± 43.6	63.8	79.0 ± 5.1
4	.1% stilb. .2 μg B_{12}	55.1	91.1	36.0	6.8	268.5	293.8 ± 26.3	67.8	73.8 ± 7.4
5	.1% stilb. .4 μg B_{12}	58.0	98.4	40.4	7.6	333.4	340.8 ± 37.7		
6	.16% thyro.‡	58.4	87.8	29.4	9.3	196.1	225.8 ± 14.6		
7	.16% thyro. .2 μg B_{12}	57.2	98.7	41.5	10.8	213.4	217.9 ± 22.1		
8	.1% stilb. .16% thyro.	55.5	67.5	12.0	7.0	176.8	258.7 ± 17.0	55.5	83.6 ± 6.8
9	.1% stilb. .16% thyro. .2 μg B_{12}	55.5	80.7	25.2	8.5	189.1	234.3 ± 16.8	61.9	76.9 ± 5.3
10	.1% stilb. .16% thyro. .4 μg B_{12}	57.8	88.0	30.2	8.9	229.7	266.0 ± 31.6		

* Standard error of mean $= \sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

† Diethylstilbestrol.

‡ Thyroprotein.

B_{12}‡ was injected subcutaneously in doses of 0.2 or 0.4 μg daily. All rats were housed in a temperature-controlled animal room at 75°F. Body weight and food intake data were collected every second day. On the 20th day the animals were killed and weighed. The testes were removed from all and the seminal vesicles from half of the rats, and weighed on a Roller-Smith balance.

Results. Body growth and food intake. The data are summarized in Table I. The average final body weights and daily food consumption of the controls (group 1) were about the same as the rats supplemented with vit. B_{12} (group 2). The diethylstilbestrol-treated rats (group 3) gained less than half the weight and consumed less than ⅔ as much food as the controls. Injections of 0.2 (group 4) or 0.4 μg of vit. B_{12} (group 5) partially counteracted the growth and appetite-depressing actions of the estrogen, with the larger dose of the vitamin showing the greater effect.

Thyroprotein (group 6) reduced growth considerably but had no appreciable effect on food intake. When vit. B_{12} was added (group 7) it partially overcame the inhibition of growth and increased the total food consumption above that of the controls. The most drastic retardation of growth occurred when diethylstilbestrol and thyroprotein were fed together (group 8). Total food consumption was intermediate between that consumed by the groups given diethylstilbestrol or thyroprotein only. Vit. B_{12} (groups 9 and 10) partially overcame these inhibitory effects of the two hormones on growth and appetite.

Testes and seminal vesicles. There were no significant differences between the testes weights of the controls (group 1) and rats injected with vit. B_{12} (group 2). Diethylstilbestrol (group 3) reduced the weight of the testes to about ⅓ of that in the controls, presumably through inhibiting FSH secretion by the anterior pituitary. The weight of the seminal vesicles was doubled on the basis of body weight, probably as the result of a direct stimulation of the seminal vesicles. Vit. B_{12} (groups 4 and 5) did not modify these actions of diethylstilbestrol on the testes or seminal vesicles.

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‡ Diethylstilbestrol and crystalline vit. B_{12} (Cobione) were kindly supplied by Dr. D. F. Green of the Veterinary Division, Merck and Co., Rahway, N. J., and thyroprotein (Protamone) by the Cerophyl Laboratories, Inc., Kansas City, Mo.

Thyroprotein (group 6) decreased the weight of the testes even to a greater degree than diethylstilbestrol. The addition of vit. B_{12} (group 7) did not alter this result. The effects of diethylstilbestrol and thyroprotein together (group 8) on the testes and seminal vesicles were also not changed by the addition of vit. B_{12} (groups 9 and 10).

Discussion. These data indicate that vit. B_{12} can at least partially overcome the growth-retarding action of diethylstilbestrol in young male rats. It is possible that a greater dose of the vitamin than used in these experiments would have completely overcome these inhibitory effects of diethylstilbestrol. The greater food consumption by the vitamin-supplemented rats was at least partially responsible for the increased growth.

Vit. B_{12} did not alter the effects of diethylstilbestrol or thyroprotein on the testes or seminal vesicles. This is in contrast to the observation of Ershoff *et al.*(7,8) that desiccated whole liver or yeast can overcome the inhibition of ovarian development in immature rats fed massive doses of alpha estradiol. It is possible that these food substances contain a factor or factors other than vit. B_{12} which are effective in this respect. Differences between the actions of natural estrogens, such as estradiol, and diethylstilbestrol(6) must also be considered.

Unfortunately, the data presented here cannot be considered as evidence for or against the view that vit. B_{12} favors the disappearance of thyroprotein or diethylstilbestrol from the body. The large amounts of diethylstilbestrol or thyroprotein fed the rats may have been more than sufficient to induce maximal responses by the testes and seminal vesicles, and hence any relatively small change in the catabolism or excretion of these hormones would not be reflected in the response of the two organs.

Monroe and Turner(9) recently reported that vitamin B_{12} may be able to increase the catabolism of administered thyroxine in baby chicks, but no effect of vit. B_{12} could be demonstrated by the senior author on normal thyroid function in rats(4) or chicks(10). Although the ability of vit. B_{12} to counteract the growth-retarding effects of large doses of diethylstilbestrol or thyroprotein in rats may be attributed in part to its stimulation of greater food intake, other channels for its effectiveness are also indicated.

Summary. The effects of crystalline vit. B_{12} on several actions of diethylstilbestrol and thyroprotein were determined in 100 young male albino rats. Diethylstilbestrol and thyroprotein were incorporated into the ration in amounts of 0.10 and 0.16% respectively, and vit. B_{12} was injected subcutaneously in doses of 0.2 or 0.4 μg daily. Vit. B_{12} partially counteracted the growth-retarding action of these two hormones when given individually or together, and also increased food consumption. The vitamins did not alter the ability of diethylstilbestrol or thyroprotein to depress testes weight or the ability of the former to increase the weight of the seminal vesicles.

Addendum. Further data obtained since this manuscript was submitted indicate that vit. B_{12} probably does not alter the turnover of thyroprotein in the rat. The increase in the basal metabolism of rats given thyroprotein was found to be unaffected by vit. B_{12} administration.

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Further Studies on Serial Passage of a Mixture of Influenza Viruses in Embryonated Eggs.* (18435)

JOHN Y. SUGG.

From the Department of Bacteriology and Immunology, Cornell University Medical College, New York.

It has been reported previously(1) from this laboratory that a mixture of the PR8 strain of influenza A virus and the Lee strain of influenza B virus was carried through 10 serial passages in embryonated eggs. In those experiments, passage was always made by the intra-allantoic inoculation of a 10^{-3} dilution of allantoic fluid, and in the fluids of all harvests both viral agents were present in sufficient concentration to agglutinate chicken erythrocytes. In the present report it will be shown that the PR8 and the Lee viruses can be maintained as a mixture through at least 52 egg to egg transfers. In these experiments passage was made by the inoculation of undiluted instead of diluted fluid and occasionally one or the other of the viruses was not detected by hemagglutination for as many as two consecutive passages, but, on the next passage, reappeared in sufficient concentration to cause hemagglutination in relatively high dilution.

Experimental. Allantoic fluids obtained from eggs inoculated 48 hours previously with either PR8 or Lee virus were combined and used as starting material. Passage was always made by inoculating 0.2 ml of undiluted allantoic fluid, to which penicillin had been added, into the allantoic sac of 2 or 3 eggs containing 10- to 12-day-old embryos. The inoculated eggs were incubated for 48 hours at 35°C, and were then chilled at 4°C for 4 to 24 hours before the allantoic fluids were harvested. The capacity of each fluid to agglutinate chicken erythrocytes in the presence of PR8 antiserum, Lee antiserum, and saline was determined. This procedure was conducted by a pattern test(2) in which equal volumes (0.2 ml) of serial 2-fold dilutions of virus suspension, antiserum or saline, and 0.5% chicken erythrocytes were combined and incubated at room temperature for approximately 1 hour. In addition, the fluids of the first 11 passages were tested in exactly the same manner against a 0.3% suspension of guinea pig erythrocytes. The latter tests were then discontinued since the results were essentially the same as those obtained with chicken cells.

Results. In Table I are shown the data obtained with the fluids used for the first 11 passages. It will be seen that there was considerable variation in the capacities of the different fluids to produce agglutination in the presence of the different antiserums. If that agglutination is an indication of the concentration of the heterologous virus particles, it is obvious that not only the concentration of the different viruses varied from passage to passage, but that the variation of the 2 agents was not always in the same direction so that the PR8/Lee ratio fluctuated over a wide range. This is well illustrated by a comparison of passages No. 2 and 3; in the first instance Lee was present in relatively high concentration whereas PR8 was barely detectable, but in the next passage (No. 3) the relationship was reversed. The data in Table I are representative of the results of the hemagglutination tests with all of the subsequent fluids; the agglutinin titers continued to rise and fall, and on several occasions one or the other of the viruses was not detected by this method during two consecutive passages, but on the next passage reappeared in sufficient concentration to produce hemagglutination in relative high dilution (*e.g.* passages 7-9). Both agents persisted through 52 egg to egg transfers, and the impression was obtained that the presence of one virus had little, if

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TABLE I. Examples of Agglutination of Chicken* Erythrocytes by Mixtures of Dilutions of Allantoic Fluids and Constant Amounts of Antiserum.

		Results 2-fold dil. of allantoic fluid									
Passage No.	Antiserum	1	2	3	4	5	6	7	8	9	10
0	PR8	++	++	++	++	++	++	++	+	0	0
	Lee	++	++	++	++	++	++	++	0	0	0
	Saline	++	++	++	++	++	++	++	++	0	0
1	PR8	++	++	++	++	++	++	++	0	0	0
	Lee	+	0	0	0	0	0	0	0	0	0
	Saline	++	++	++	++	++	++	++	++	0	0
2	PR8	++	++	++	++	++	++	++	0	0	0
	Lee	+	0	0	0	0	0	0	0	0	0
	Saline	++	++	++	++	++	++	0	0	0	0
3	PR8	0	0	0	0	0	0	0	0	0	0
	Lee	++	++	++	++	++	0	0	0	0	0
	Saline	++	++	++	++	++	++	0	0	0	0
4	PR8	++	++	+	0	0	0	0	0	0	0
	Lee	++	++	++	++	++	++	++	++	++	0
	Saline	++	++	++	++	++	++	++	++	++	+
5	PR8	++	++	++	++	++	0	0	0	0	0
	Lee	++	++	++	++	+	0	0	0	0	0
	Saline	++	++	++	++	++	++	++	0	0	0
6	PR8	++	++	++	++	++	++	++	++	0	0
	Lee	++	+	0	0	0	0	0	0	0	0
	Saline	++	++	++	++	++	++	++	++	++	0
7	PR8	++	++	++	++	++	++	++	0	0	0
	Lee	0	0	0	0	0	0	0	0	0	0
	Saline	++	++	++	++	++	++	++	0	0	0
8	PR8	+	0	0	0	0	0	0	0	0	0
	Lee	0	0	0	0	0	0	0	0	0	0
	Saline	+	0	0	0	0	0	0	0	0	0
9	PR8	0	0	0	0	0	0	0	0	0	0
	Lee	++	++	++	++	++	++	++	++	0	0
	Saline	++	++	++	++	++	++	++	++	0	0
10	PR8	++	++	+	0	0	0	0	0	0	0
	Lee	++	++	++	++	++	++	++	++	+	0
	Saline	++	++	++	++	++	++	++	++	++	+
11	PR8	++	++	++	++	++	++	+	0	0	0
	Lee	++	++	++	++	++	+	0	0	0	0
	Saline	++	++	++	++	++	++	++	++	0	0

++, +, and 0 complete, partial, and no agglutination, respectively.

* In parallel tests with 0.3% guinea pig RBC all the fluids gave essentially the same results as those presented for the chicken RBC.

any influence upon the multiplication of the other.

However, a rise in hemagglutinins is not always accompanied by a corresponding rise in infectivity when undiluted allantoic fluid is used as inoculum(3,4). Therefore, in order to obtain more information on the relative proportions of the two viruses, the egg-infectious titers were determined for a number of the dually infected fluids when combined with PR8 or with Lee antiserum. For these tests, equal volumes of serial 10-fold dilutions of the test fluids, prepared in infusion broth, and of a 1:15 dilution of antiserum were mixed, and sufficient penicillin was added to provide 100 units per ml. After the mixtures had been incubated 30 minutes at 37°C, 0.2 ml of each mixture was inoculated into the allantoic sac of each of 3 9- to 12-day-old embryonated eggs. The inoculated eggs were incubated for 48 to 72 hours at 35°C and were then tested for hemagglutinins; in the

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TABLE II. Comparison of Egg-infectious Titer and Hemagglutinating Capacity of Different Allantoic Fluids When Mixed with PR8 or Lee Antiserum.

Allantoic fluid from passage No.	Antiserum added to test	Egg-infectious titer	Hemagglutinating capacity (chicken erythrocytes) 2-fold dil. of allantoic fluid									
			1	2	3	4	5	6	7	8	9	10
44	PR8	4.3*	0	0	0	0	0	0	0	0	0	0
	Lee	7.0	++	++	++	++	++	0	0	0	0	0
	Saline	†	++	++	++	++	++	+	0	0	0	0
45	PR8	6.3	++	++	++	++	+	0	0	0	0	0
	Lee	6.7	++	++	++	++	++	++	++	++	0	0
	Saline	†	++	++	++	++	++	++	++	++	++	0
46	PR8	5.1	++	++	++	++	++	++	+	0	0	0
	Lee	6.3	++	++	++	++	++	++	0	0	0	0
	Saline	†	++	++	++	++	++	++	++	++	0	0
47	PR8	2.5	++	++	++	++	++	+	0	0	0	0
	Lee	4.5	0	0	0	0	0	0	0	0	0	0
	Saline	†	++	++	++	++	++	++	0	0	0	0
48	PR8	4.8	0	0	0	0	0	0	0	0	0	0
	Lee	4.5	0	0	0	0	0	0	0	0	0	0
	Saline	†	0	0	0	0	0	0	0	0	0	0
49	PR8	4.5	++	++	++	++	++	++	0	0	0	0
	Lee	8.5	++	++	++	++	++	++	++	+	0	0
	Saline	†	++	++	++	++	++	++	++	++	++	0
50	PR8	3.0	++	++	++	++	++	0	0	0	0	0
	Lee	6.8	++	++	++	++	++	++	++	+	0	0
	Saline	†	++	++	++	++	++	++	++	++	++	0
51	PR8	3.0	0	0	0	0	0	0	0	0	0	0
	Lee	5.3	++	++	++	++	++	++	0	0	0	0
	Saline	†	++	++	++	++	++	++	++	+	0	0
52	PR8	6.8	++	++	++	++	++	++	++	0	0	0
	Lee	5.5	++	++	++	++	++	0	0	0	0	0
	Saline	†	++	++	++	++	++	++	++	++	0	0

* Reciprocal of the log of 50% end point.
† No test.

absence of agglutination, the eggs were considered free from infection. The titers are expressed as the reciprocal of the log of the 50 per cent end point as determined by the method of Reed and Muench(5).

The data in Table II show that the PR8/Lee ratios differed greatly in the various fluids when determined by egg-infectious titer, as well as when determined by hemagglutination. However, the results obtained by the two tests did not always correspond. For example, in passage 47 the infectivity tests indicated a higher concentration of PR8 than of Lee, whereas the hemagglutination tests indicated the opposite relationship. But the ratio of the 2 viruses in the inoculum, as determined by either method, was not an indication of the relative concentrations of the agents that would be present in the harvested allantoic fluids. This is particularly well illustrated by a comparison of passages 50 and 52. In the former instance, both tests indicated a higher concentration of PR8 than of Lee in the inoculum (passage 49), and the same relationship was found in the harvest (passage 50); whereas in the latter instance, both tests again showed a higher PR8/Lee ratio in the inoculum (passage 51), but there was a definitely lower concentration of PR8 than of Lee in the harvested fluid (passage 52). It also will be seen that in some passages the drop in virus activity was greater than could be accounted for on the basis of dilution alone, but whether or not either agent failed to multiply during any one passage cannot be determined with certainty from the present data. Nevertheless, sufficient multiplication did occur to maintain the mixture through 52 consecutive egg passages, and at the end of

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the experimental period each agent was recovered in an apparently pure culture from eggs that had been inoculated with the dually infected allantoic fluid plus either PR8 or Lee antiserum.

Discussion. The results of previous experiments(1) had indicated that a mixture of the PR8 strain of influenza A virus and the Lee strain of influenza B virus could be maintained indefinitely by serial passage in embryonated eggs, and the present report has provided additional evidence in support of that conclusion. This finding is in contrast to what might be expected. In the chick embryo PR8 has a faster growth rate than Lee(6) and under proper conditions the two viruses reciprocally interfere with the multiplication of each other(7), and it might be expected that Lee would have been eliminated on serial passage. Hence, the continued persistence of the two viruses as a mixture raises the question as to whether or not the same host metabolic systems that are required for the multiplication of PR8 are likewise required for the multiplication of Lee. In that connection it is significant that the propagation of the mixture has been accomplished by the use of diluted(1) as well as undiluted allantoic fluids. It is well known that the latter method does not produce maximum virus multiplication, and, although this did not alter the final results (*i.e.*, the successful passage of the mixture), it may have been responsible in large part for the pronounced differences observed among the PR8/Lee ratios of the various fluids.

Recent observations(3,4,8) indicate that if high concentrations of influenza virus are used as inocula, one can obtain what appear to be "immature" virus particles that are noninfectious, but that are capable of agglutinating erythrocytes and of interfering with multiplication of active virus. In all probability the concentrations of both PR8 and Lee particles of that sort varied greatly from passage to passage; when the concentration was high there was little if any multiplication of the homologous virus, but when the concentration was low (due to dilution on additional passage) the virus again multiplied to high titer, and a new cycle was started. But, in that event, the "immature" PR8 and Lee particles must have been more effective in interfering with the multiplication of one (presumably homologous) virus than the other, since it is obvious that the rise and fall in the concentrations of the two viruses did not always parallel, whether based on hemagglutination or on egg-infectious titer.

Summary. Undiluted allantoic fluids were used as inocula for serial passage of a mixture of the PR8 and Lee strains of influenza virus in embryonated eggs. Although wide differences were found in the relative concentrations of the two viruses in the various fluids, the mixture was successfully carried through 52 consecutive passages, and each agent was recovered in an apparently pure culture at the end of the experimental period. Since the two viruses have different growth rates and are capable of interfering with the multiplication of each other, the persistence of both agents during serial passage of the mixture suggests that multiplication of the PR8 and of the Lee viruses may not require the same host factors.

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Citrovorum Factor and Oviduct Response to Stilbestrol in Aminopterin-Treated Chicks. (18436)

IRENE T. KLINE AND RALPH I. DORFMAN.

From the Departments of Biochemistry and Medicine, Western Reserve University, School of Medicine and Lakeside Hospital, Cleveland, O.

Response of the chick oviduct to stilbestrol is drastically limited by a deficiency of pteroylglutamic acid (PGA), whether the deficiency be produced by feeding a PGA-deficient diet or by administering PGA antagonists(1-4). There is, furthermore, a quantitative relationship between the response of the chick oviduct to injected stilbestrol and the dietary level of PGA or the administered dose of aminopterin, a highly potent antagonist of PGA(2,4). Protection against aminopterin interference with stilbestrol action on the chick oviduct is afforded by PGA administered one hour prior to aminopterin in a ratio of PGA to antagonist of 300:1(2). Failure of high doses of PGA to prevent the toxic effects of aminopterin in several species has been reported(5-9). In mice, however, positive protection occurs with a ratio of PGA to aminopterin of 500:1(10). More efficient than PGA in preventing aminopterin is the factor (CF) essential to *Leuconostoc citrovorum*(11-13,15), a substance which has been shown to be related to PGA by chemical(13) and metabolic(14) synthesis from PGA. Nichol and Welch(15) found that 250,000 units of CF counteract the toxic effects of 25 μg of aminopterin in rats deprived of a dietary source of PGA for 7 days; in mice, about 20,000 units of CF were required to overcome 1-4 μg of aminopterin, according to the work of Broquist *et al.* as reported by Cravens and Snell(12); while for *L. citrovorum* only 11 units of CF were required per 1-4 μg of aminopterin(16); in the chick embryo, as high as 6 million units partially counteract 20 μg of aminopterin(12).

The data presented below indicate that, under the experimental conditions employed, CF prevents aminopterin inhibition of stilbestrol stimulation of the chick oviduct in a ratio of vitamin to antagonist of about 16:1. Under the same conditions PGA in a ratio of 500:1 is 81% as effective in preventing aminopterin inhibition.

Materials and methods. Two-day-old White Leghorn pullets obtained from Kerr Chickeries, Frenchtown, N. J., were arranged in 16 groups as indicated in Table I and were given their first injections immediately upon arrival in the laboratory. They were thereafter housed in electrically heated brooders with raised, wire-mesh floors. No food was allowed but tap water was provided *ad libi-*

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TABLE I. Effect of Citrovorum Factor on Aminopterin Inhibition of Stilbestrol Stimulation of the Chick Oviduct.

	Injections						Autopsy results		
Group No.	No. of chicks	PGA total dose, 22.5 mg	CF total dose, µg	4NH$_2$-PGA, total dose, 15µg	Stilbestrol in corn oil, total dose, 2 mg	Corn oil only	No. of survivors	Mean body wt, g ± S.E.*	Mean oviduct wt, mg ± S.E.
1	10	0	0	0	0	+	10	36.2 ± 0.9	11.2 ± 1.5
2	12	0	0	+	0	+	11	33.5 ± 0.7	11.5 ± 0.9
3	10	0	0	0	+	0	9	33.0 ± 0.9	63.9 ± 3.5
4	26	0	0	+	+	0	17	33.4 ± 0.5	23.4 ± 0.8
5	10	+	0	0	0	+	9	32.1 ± 0.8	15.9 ± 1.2
6	10	+	0	+	0	+	9	31.2 ± 0.9	13.9 ± 0.9
7	10	+	0	0	+	0	10	31.8 ± 0.6	73.8 ± 4.1
8	10	+	0	+	+	0	10	31.7 ± 0.9	60.4 ± 2.7
9	10	0	30	0	0	+	9	32.3 ± 0.8	14.3 ± 0.8
10	12	0	30	+	0	+	12	32.5 ± 0.7	12.8 ± 1.0
11	10	0	30	0	+	0	10	33.0 ± 1.1	71.4 ± 4.1
12	20	0	30	+	+	0	20	33.8 ± 0.5	39.5 ± 1.8
13	9	0	240	0	0	+	7	30.0 ± 0.6	12.6 ± 1.2
14	12	0	240	+	0	+	12	33.5 ± 0.8	13.4 ± 0.6
15	10	0	240	0	+	0	10	33.0 ± 0.5	61.5 ± 1.6
16	20	0	240	+	+	0	20	35.7 ± 2.0	55.1 ± 2.5

* Standard error of the mean.

tum. The complete injection schedule was as follows:

Day	*Time*	*Injections*
1	4:00 P. M.	PGA, CF or saline
	5:30	Aminopterin
2 and 3	8:30 A. M.	PGA, CF or saline
	10:00	Aminopterin and Stilbestrol (or oil)
4	8:00-12:00	Autopsy

Dosages were, per dose:

PGA: 7.5 mg in 0.5 ml solution (Folvite[1]).

CF[1]: 10 µg or 80 µg in 0.5 ml of 0.9% saline.

Aminopterin:* 5 µg in 0.1 ml of 0.9% saline.

Stilbestrol:† 1 mg in 0.1 ml of corn oil.

Controls: 0.5 mg of 0.9% saline was used as the control solution in chicks receiving neither PGA nor CF, while 0.1 ml of corn oil was administered to those receiving no stilbestrol.

At autopsy, the chicks were killed with chloroform and weighed. Oviducts were then immediately dissected out, blotted, and weighed on a Roller-Smith torsion balance to the nearest milligram.

Results. In newly-hatched chicks subsisting on their own body stores‡ for 5 days, 2 mg of stilbestrol stimulated a 5.5 fold increase in oviduct weight in 48 hours (Table I). Aminopterin (15 µg total dose) prevented approximately two-thirds of the stilbestrol-induced proliferation but did not reduce the sensitivity of the oviduct to endogenous estrogen.

In PGA-supplemented chicks a significantly greater oviduct growth occurred even though body weight decreased significantly (Tables I and II). This may indicate greater ovi-

TABLE II. Statistical Comparisons of Mean Oviduct Weights.

Groups compared	t*	P
1 and 5	3.1	<0.01
3 ″ 7	1.85	0.1-0.05
3 ″ 11	1.25	0.3-0.2
11 ″ 15	1.96	0.1-0.05
15 ″ 16	0.51	>0.5
7 ″ 8	2.85	0.02-0.01

* Fisher's t value(17).

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*Pteroylglutamic acid (Folvite), aminopterin and Citrovorum Factor Concentrate were generously supplied by the Lederle Laboratories Division of the American Cyanamid Co. through the courtesy of Dr. T. H. Jukes. According to information received from Dr. Jukes the Citrovorum Factor Concentrate (Leucovorin, synthetic Citrovorum Factor) made available to us contained 20 Million Units/ml. Five µg represent about 25000 Units.

† Stilbestrol was kindly made available by Winthrop-Stearns, Inc, Inc.

‡ The caloric value of the injected corn oil was 772 small calories per 0.1 cc oil.

duct sensitivity to, decreased inactivation of or increased secretion of endogenous estrogen. Aminopterin, at the dose employed, did not interfere with the endogenously increased oviduct growth.

When stilbestrol was administered to PGA-supplemented chicks, in the absence of aminopterin treatment, oviduct proliferation was significantly greater than in non-supplemented chicks. The increase in oviduct weight was 6.5 fold in comparison to Group 1 and 4.5 fold with reference to Group 5. Aminopterin, under the conditions used, interfered only to the extent of 19% with the action of stilbestrol in the PGA-treated chicks. That is to say, in the time intervening between the injection of PGA and the injection of aminopterin, the conversion of PGA to its metabolically active form(15) was adequate to permit 81% of the possible stilbestrol action. If aminopterin is also antagonistic to the active form of PGA(15) possibly more conversion occurred than is evident in the data.

Citrovorum factor (CF) in a total dose of 30 μg permitted growth of the oviduct both in the absence and presence of exogenous estrogen as well as did a total dose of 22.5 mg of PGA but was not as effective in preventing the action of aminopterin (total dose of 15 μg). When the total dose of CF was increased to 240 μg, no greater stilbestrol action was permitted than occurred with the lower dose of CF or the massive dose of PGA but the higher dose of CF did effectively prevent aminopterin interference with stilbestrol action on the oviduct.

Summary. Data have been presented which show that Citrovorum Factor (CF) in a ratio to aminopterin of approximately 16:1, but not of 2:1, counteracts aminopterin inhibition of stilbestrol stimulation of the chick oviduct, while pteroylglutamic acid (PGA) in a ratio to aminopterin of 500:1 is 81% effective in this regard. That CF can substitute for PGA in permitting stilbestrol stimulation of the chick oviduct is further evidence consistent with the theory that CF is a metabolically active form of PGA.

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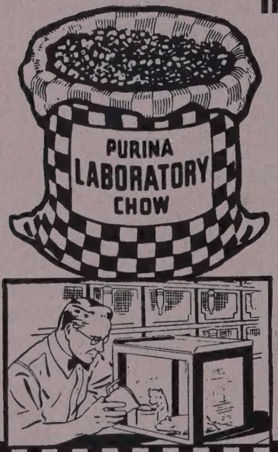

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